CAROTENOIDS UPTAKE AND METABOLISM IN THE RETINA

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List of abbreviations

AA : Ascorbic acid

min : minute

ml : millilitre

SRF : subretinal fluid

RD : retinal detachment

HPLC : High pressure liquid chromatography

$\mathbf{A_{max}}$: Absorption maximum

$\mathbf{T_r}$: retention time

g : gram

mg : milligram

μg : microgram

ng : nanogram

μl : microlitre

UV : ultraviolet

wt : weight

Abstract

A high pressure liquid chromatography method was established to analyse carotenoids in specimens from human and rats. The method utilises an isocratic elution of specimens from a silica column, using 16% dioxane in hexane.

The selected method demonstrated that the major carotenoid in human serum is ß-carotene, while lutein is the major carotenoid in subretinal fluid. The results are consistent with the current knowledge on the occurrence of ß-carotene in human serum and lutein in the retina.

On the other hand, the selected method showed negligible carotenoid in serum, liver and retina of rats. The absence of carotenoids in rat serum and retina accounts for part of the reason for the high sensitivity of rat's retina to photic injury.

Although feeding lutein or astaxanthin did show the appearance of the respective carotenoid in some specimen of the blood or tissue, the concentration was in the lower limit of our analytical method. This concentration was <1% of the values observed in human serum. Others have demonstrated the protective effect of lutein and astaxanthin against photic injury to rat's retina. These results indicate that the high potency of carotenoids because only a trace amount of them is needed to protect the retina from photic injury. Alternatively, the excessive amount of orally administered carotenoids might preserve the efficiency of other endogenous antioxidants in the retina.

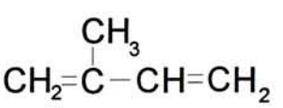
I. INTRODUCTION

A. Current knowledge on carotenoids:

1. Chemistry of carotenoids: Carotenoid was isolated from carrot in 1831. The structure was not clearly elucidated until 1925. Chromatographic technique was used to isolate and identify these pigments (Fruton & Simmonds, 1958).

Carotenoids are consisted of a long hydrocarbon backbone with a series of conjugated double bonds. The basic structure is made up of eight isoprene units. Each isoprene molecule has five carbon atoms as shown below. The main stem is formed by joining the two ends of the isoprene after the shifting of the double bond to the centre (Weedon & Moss, 1995).

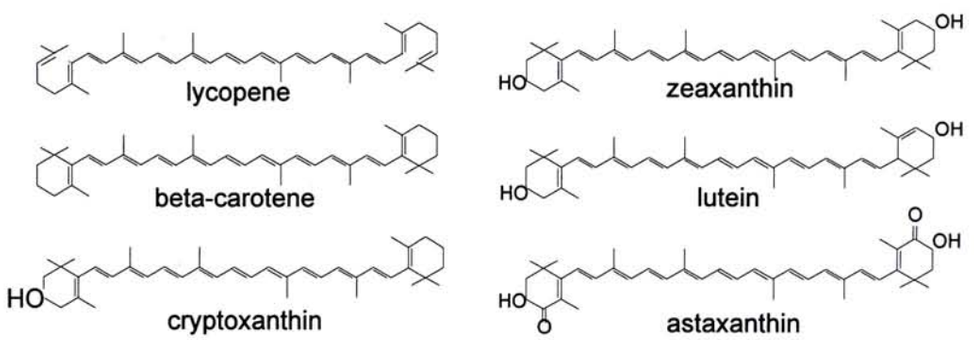
Although the number of carbon in the chain may vary slightly, the C_{40} backbone is considered as the main stem of the structure in carotenoids (Britton, 1995). Lycopene and β-carotene are two typical examples of carotenoids with C_{40} backbone. The addition of one or more isoprene units leads to the formation of larger carotenoids such as decaprenoxanthin or sarcinaxanthin (Ferezou, 1992; Weedon & Moss, 1995). In some cases when C_{40} carotenoids are shortened by degradation, they are called apocarotenoids such as capsorbin and capsanthin. Other chemical reactions such as hydrogenation, dehydrogenation, cyclization and oxidation will modify the basic structure of



isoprene

carotenoids (Pfander, 1992; Britton, 1995). These chemical processes enable the diversification of these natural pigments.

The hydrocarbon-containing carotenoid is called carotene such as lycopene, β-carotene and torulene. Carotenoids containing one or more oxygen group are known as xanthophylls (Nussbaum et al, 1981; Armstrong, 1996). Xanthophyll includes a major series of carotenoids. Lutein, astaxanthin and canthaxanthin are some widespread xanthophylls found in the world (Latscha, 1990). Some of their structures are shown in the following.



Besides the variation of different functional groups present in various carotenoids, these pigmented molecules exist in a great variety of isomer forms. The occurrences of geometric isomers of each double bond are possible (Britton, 1995). Normally, the trans isomer is the most stable configuration (Handelman, 1996).

Due to the long, linear and conjugated carbon chain, carotenoids have a specific absorption characteristic in the visible spectrum. Most carotenoids have a typical absorption maximum (A_{max}) in the visible spectrum shown in figure 1b to 3b. The triple peaks enable the spectrophotometric recognition of the carotenoids. The A_{max} of a carotenoid is resulted from the transition of electron from the lowest vibrational level of

the electron at ground state to the lowest vibrational level of the excited state (Kohler, 1995).

The location, shape and intensity of the A_{max} depend on a number of factors: (a) solvent effect (b) length of the chromophore (c) presence of cyclic ring (d) geometric isomer and (e) functional groups. The higher the refractive index of the solvent, the greater the A_{max} of the carotenoid. The length of the chromophore is determined by the number of conjugated double bonds (Englert et al, 1971; Kohler, 1995); the longer the polyene chain, the longer the wavelength in the absorption maximum. Cis isomer leads to a mild decreased absorbance and hypsochromic shift (displacement of A_{max} to short wavelength) of the A_{max}. The most frequent form of cis-isomer exists in the double bond near the centre of the molecule (Britton, 1995). The fine structure will also affect the spectral properties. For example, addition of carbonyl group will cause bathochromic shift of the A_{max} (displacement of A_{max} to long wavelength) while the presence of epoxide group leads to an hypsochromic shift of the A_{max} (Englert et al, 1971).

Although there is a predictable trend for the absorption characteristics of the spectrum, it is impossible to identify the detail structure of a carotenoid by the absorption spectrum alone.

The chemical structures of commonly occurring carotenoids have been thoroughly studied. Because of the improvement of chromatographic techniques, new carotenoids are being discovered continuously.

2. Occurrence of carotenoids: Carotenoids are the most diversified natural pigments found in the animal and plant kingdoms (Latscha, 1990; Goodwin, 1992).

These pigments enrich the colour of the world. More than 600 types of carotenoids are distributed among the plant and animal kingdom (Straub, 1987). Most carotenoids are synthesised by green plants and green algae. Some of them are synthesised by bacteria or yeast. However, animals cannot synthesize carotenoids.

(a) Micro-organisms: Carotenoids are found in both photosynthetic and non-photosynthetic bacteria. The purple and green bacteria are another subtypes of photosynthetic bacteria. Their carotenoids are located in the chromatophores of the cell membrane. In non-photosynthetic bacteria such as archebacteria (e.g. *Halobacterium*) and enbacteria, the carotenoids have a carbon chain of C_{30}, C_{45} or C_{50} rather than the usual C_{40} carotenoids found in plants and animals. The carotenoids are deposited in the cytoplasmic membrane.

(b) Plants: Higher plants have highly extensive and tissue-specific carotenoids. The pigments are located in two essential positions with respect to its functions. The first one is chloroplast in which photosynthesis takes place. When the chloroplast disintegrates, carotenoids are released into the cytoplasm and then the photosynthetic tissue changes to yellow. That is the major colour of the withered leaves in autumn (Latscha, 1990). The second location for the accumulation of carotenoids is the reproductive organ such as fruit, pollen or petals in chromoplast. Some reproductive organs of higher plants do not have carotenoids. They are species specific. The variations of colour in fruit and flowers' petals are caused by the presence of unique carotenoids.

In lower plants, such as algae, the chloroplasts are the major source of carotenoids. Their synthetic pathway resembles that of higher plants. The carotenoids of

both higher and lower plants are attached to the protein of the chloroplast membrane. During reproduction, carotenoids may be stored outside the chloroplast in lipid vacuoles. Microalgae such as *Haematococcus* is a very common algae found in nature. It accumulates β-carotene, lutein and astaxanthin ester in the cytoplasm.

(c) Animals: They cannot make their carotenoids. Carotenoids are derived from the diet. On the other hand, carotenoids are not absorbed by all animals. The occurrences of carotenoids in animals are species specific as well as tissue specific (Latscha, 1990).

Astaxanthin is widely distributed in marine animals such as sponges, crustaceans or fish. Beside astaxanthin, fish also contain lutein and zeaxanthin. Birds accumulate lutein, astaxanthin and canthaxanthin in their feathers, legs, feet or comb (Hudon et al,1992). Within the animal kingdom, carotenoids are relatively low in mammals. ß-carotene accumulated by cattle, horse and wapiti but not in sheep, pigs or cat (Latscha, 1990). Primate is one of the mammals that could store a considerable amount of carotene and xanthophyll.

(d) Human retina: The presence of non-bleaching yellow pigment in the human retina has been noted since 1782 (Nassumm et al 1981). These macular pigments are distributed in the outer and inner plexiform layers in human and monkey (Snodderly et al 1979; 1984; 1995).

Lutein is the major pigment in the retina (Nassumm et al 1981). Several studies (Bone et al, 1985; Handelman, 1988) described the presence of two oxygenated carotenoids, lutein and zeaxanthin, in human retina using chromatographic and mass

spectrometric method. Similar results were found in monkeys (Handelman, 1991; Snodderly, 1991). Zeaxanthin is concentrated at the macular region while lutein is dominant in the peripheral region of the retina.

(e) Serum: Although mammals are relatively poor in accumulating carotenoids (Latscha, 1990; Krinsky et al, 1990), some reports (Kelly et al, 1950; High et al, 1951; Shapiro et al 1984) describe that rodent tissues contain carotenoids only after long term administration with a high dosage. Shapiro et al (1984) studied the uptake of β-carotene in Sprague-Dawley rats. The dosage of 150 mg/kg was used for 21 weeks in order to record an obvious level of β-carotene in serum and other tissues.

On the other hand, the concentration of carotenoids in the primate is obviously higher than that in the rodent (Krinsky, 1990; Snodderly, 1990). The variations of carotenoids in monkey and human were large. In human, the standard deviation could be half to two third of the mean value (Carughi, 1993; Mares-Perlman et al, 1995; Kostic et al 1995). It indicates that the mechanism of uptake is multifactorial.

3. Metabolism of carotenoids: Most of previous metabolic studies are concentrated on β-carotene. The metabolism of carotenoids involved the degradation and structural modification of the molecules. That involves the catabolic breakdown to apocarotenoid, epoxycarotenoids or retinoids. The oxidation of the immediate products can eventually degrade to CO_2 and H_2O. In animals, β-carotene is the most widely studied carotenoid in the metabolic process. Pro-vitamin A activities received the greatest attention.

In plants, ripening of fruit is an example of carotenoid metabolism. During ripening, chlorophyll and carotenoids are released from the chloroplast into the cytoplasm. Carotenes are degraded to apocarotenoids and further oxidized to terpenoid products or even CO_2 and H_2O. On the other hand, xanthophyll are esterified with fatty acids and persist in the cytoplasm. In the fruit of paprika, zeaxanthin is converted to capsanthin and capsorubin. The whole process involves the change from green to yellow and finally to a red colour product.

In animals, Oslon (1993, 1994) demonstrated the cleavage of β-carotene at the centre of the molecule. The formation of retinal from β-carotene was found in both the liver and intestine homogenate of rats. Other carotenoids such as astaxanthin, canthaxanthin also have pro-vitamin A activities (Latscha, 1990; Schiedt, 1985). Retinol is esterified with fatty acid in the human intestine and is transported to the liver by chylomicrons (Erdman et al, 1993; Parker, 1995).

Other catabolic products such as β-apo-8'-carotenal, β-apo-13'-carotenal were found in the ferret or human intestine homogenate. All these studies indicate central and random cleavage of the provitamin A carotenoids or β-carotene in the metabolic pathway (Olson, 1993; Parker, 1995).

Krinsky (1993) demonstrated the *in vitro* conversion of β-carotene to retinyl ester in rats and monkeys. He suggested the species variation in provitamin A activities. *In vivo* study in human, rats and ferrets confirmed that the dietary supplement of β-carotene resulted in retinyl ester and unmetabolised β-carotene (Weiser et al, 1993; Vliet et al, 1995; Rock et al, 1996).

4. Biological function: The most well-known function of the pigments is the colour signal produced for communication and species recognition of both animals and plants. The colour and pattern of carotenoids could be expressed as attraction, protection or warning signal (Latscha, 1990).

In plants, the biological functions are studied in more detail comparing to that of animals (Davies, 1985). Carotenoids can absorb light and transfer the excited energy to the chlorophyll for photosynthesis (Olson, 1992; Schalch, 1992; Krinsky, 1994;). They protect plant tissues against photoxidation when the chlorophyll is excited to its triplet state. Similarly, carotenoids quenched the energy in a singlet oxygen during the photochemical process. These pigments can work as light energy conductor due to the π-elections in the conjugated double bond (Latscha, 1990).

The function of carotenoids in animals is based on the study with β-carotene. In animals, pro-vitamin A activity is the well-known function (Krinsky, 1993). The series of conjugate double bonds enable carotenoids to function as an antioxidant (Liebler, 1993; Olson, 1994; Sies, 1995). Carotenoids may work by its own action or preserve other antioxidants in the biological system. *In vitro* studies have been used to quantify the bleaching effect or oxidation products after the addition of carotenoid (Burton, 1984; Miki, 1991; Chopra et al, 1993). Animals study using rats, chicken and guinea pigs demonstrated the decrease in lipid peroxidation after taking β-carotene, astaxanthin or canthaxanthin (Palozza, 1992).

Some carotenoids such as β-carotene and canthaxanthin affect the gene expression of the protein connexin 43. This protein can promote the communication of

adjacent cells via the gap junction (Bendich, 1993). Other possible actions of the carotenoids are: (1) Maintenance of the normal reproduction process in bird, cattle, horse and pig, (2) Fertilization and growth in fish and crustacean (Latscha, 1990).

The role of carotenoids in the retina is not fully understood (Berman, 1991; Gerster, 1991). Several epidemiological studies (Goldberg et al, 1988; Seddon et al, 1994; Richer et al, 1996) reported that carotenoids were deficient in eye diseases such as age-related macular degeneration and cataract. However, Mares-Perlman et al (1995) did not find any strong association between carotenoids level in the blood and the incidence of age-related macular degeneration.

Since the pigments distributed in the inner and outer plexiform layer of the neuroretina, they were believed to screen out blue light with its absorption effect (Nassbaum et al, 1981; Marshall, 1985; Schalch, 1992). Monkeys taking carotenoids deprived diet demonstrated changes in the integrity of retina (Malinow et al, 1980). Relationship between age-related macular degeneration and macular pigment had been proposed (Goldberg et al, 1988; Snodderly, 1995). The actual mechanism had not been elucidated yet.

Protective effect of carotenoids against light injury to rat retina has been demonstrated by Tso (1989) and Lam (1996). Photic injury is related to free radical formation during exposure to light. The exact mechanism is not yet known.

B. Statement of problem

As reviewed above, others have postulated a role of carotenoids as an effective antioxidant. Among different kinds of carotenoids, astaxanthin has the highest antioxidant activity tested *in vitro*. However, it is not certain if astaxanthin has the same potency *in vivo* as effective as the results indicated in the *in vitro* experiments. On the other hand, lutein is the major carotenoid in human retina. Therefore, astaxanthin and lutein are selected for the present study.

Rat is a convenient animal model to study photic injury to the retina. Light-induced free radical damage to rat's retina has been studied thoroughly in the past. However, the absorption and metabolism of lutein and astaxanthin by rats are not known. In order to use rats as an animal model to assay for the biological potency of carotenoids against photic injury, it is important to know the ability of rats to utilise carotenoids in the diet.

This study began with the selection of a chromatographic method for the analysis of carotenoids. The selected method was used to analyse carotenoids in human serum and subretinal fluid to assure that the selected method produces data comparable to the current knowledge of carotenoids. The selected method was used to study carotenoids in rats' liver, serum and retina in order to assess the value of rat as an animal model to study the potency of carotenoids against photic injury.

II. MATERIAL AND METHODS

A. Material

1. Animals: Eighty four male Lewis rats were used in the carotenoids feeding experiment. They were supplied by the Animals House of the Chinese University of Hong Kong. The age of the animals was 40 to 50 days old. The animals were kept in cages with three to four animals in each cage. A normal carotenoid-free diet (Prolab RMH 2500, PMI Feeds Inc., St. Louis, MO) was given every day. All animals were kept in 12 hours cyclic light for at least 1 weeks before the feeding of carotenoids. The dark cycle sustained at an average of 0.1 to 0.6 foot-candle while 46 to 55 foot-candle in the light cycle. The temperature was maintained between 20 and 23° C. The relative humidity of the animal room was kept between 48 to 60 %.

2. Human samples:

(a) Serum: Thirteen samples of human serum were collected from TODAY X-Ray & Medical Laboratory Company Limited in Kwun Tong. The blood was drawn for a routine medical examination. The patients had no known disease. The samples were extracted with ethanol and hexane for three times. The extracts were combined, redissolved in 50 μl of dioxane in hexane (V/V: 16/84). 40 μl was used for HPLC analysis.

(b) Subretinal fluid: Six patients with rhegmatogenous retinal detachment were studied. They are patients in Tung Wah East Hospital. Three patients had acute superior bullous detachment, two patients had chronic inferior retinal detachment, one

had total retinal detachment with grade C proliferative vitreoretinopathy. The patients were treated by scleral buckling, cryopexy and drainage of subretinal fluid. An aliquot of 150 to 250 μl of subretinal fluid was obtained during the operation. Blood samples of the above patients were also collected at the time of surgery.

3. Chemicals:

(a) Standards for HPLC: All trans-retinol acetate, retinal, retinoic acid, retinol, ß-carotene and ascorbic acid were supplied by Sigma Chemical Company, St. Louis, MO; lutein, zeaxanthin, cryptoxanthin by Indofine Chemical Company, Belle Mead, NJ; lycopene, by Markhteshim Chemical Works, Ltd. Beer-Sheva, Israel.

(b) Carotenoids supplement: Carophyll Pink (F. Hoffmann-La Roche Ltd. AG-4002 Basel, Switzerland) was one kind of granulated beadlets in which the synthetic astaxanthin was distributed in a starch-encapsulated gelatine and carbohydrate matrix. The diameter of the beadlets varied from 0.15 to 0.4 mm(Latscha, 1990).

Yellow Pixafil (Industrias Alcosa, S. A. de C.V.) is a source of lutein derived from marigold petals in powder form and was used as a supplement for poultry diet. The pigment was kept at 4°C in opaque aluminium bags.

4. High Pressure Liquid Chromatography apparatus

(a) HPLC apparatus: Waters 600 LC system, Waters 996 photodiode array detector were used in all HPLC analysis.

(b) Columns: Nova PAK silica columns (3.9x300 mm, 3.9x150 mm) were supplied by Waters Associate.

(c) Solvent: Hexane and ethanol was supplied by Riedeel-de Haeen, Germany; Dioxane (1-4-Dioxan Chromasolv), by Fluka Chemical, Buch; 10mM ammonia phosphate by Sigma Chemical Company, St. Louis, MO.

B. Methods

1. Animal specimens

(a) Carotenoid supplement: Carophyll Pink (F. Hoffmann-La Roche Ltd. AG-4002 Basel, Switzerland) was grounded in a porcelain mortar for 5 minutes. The sample was suspended in olive oil (Bertolli Inc., milano, Italy) to a final concentration of 1mg/ml or 10 mg/ml astaxanthin. Yellow Pixafil (Industrias Alcosa, S. A. de C.V.) was suspended in olive oil (Bertolli Inc., milano, Italy) to a final concentration of 1 mg/ml, 10 mg/ml or 30mg/ml. One ml of carotenoid containing oil was fed to rats. The sample was fed to each rat through a stomach tube, once a day for 6 days. Two kind of control animals were used. A control group was kept under normal diet (n=10). The second group of control rats (n=34) were fed in the same manner as the treated group with olive oil.

(b) Tissue preparation: The rats were euthanized 16 hours after the last feeding of carotenoids. Deep anaesthesia was maintained by intraperitoneal injection of nembutal (40 mg/kg bodyweight). The animals were dissected just below the thoracic cavity and 5 to 8 ml of venous blood was obtained by heart puncture at the right auricle. The rats were perfused with about 150 ml of saline and perfusion was ensured by the whitening of the fundus.

Retina: The eyes were enucleated. The globe was dissected at the limbus. The lens was removed, and the retina was removed by a pair of forceps. The attachment to the optic nerve was cut by a pair of scissors. The tissue was weighed and stored at -84°C.

Serum: The blood samples were allowed to coagulate at room temperature for 15 to 20 minutes. Serum was separated from the blood cells by centrifugation at 7000xg for 10 minutes. The samples were stored at -84°C before carotenoid analysis.

Liver: The lower end in the right lobe of the liver was excised after perfusion. The samples were kept at -84°C for later analysis.

2. Human specimens: About 5 ml of venous blood was collected from each patient at the time of retinal detachment surgery. After coagulation, the samples were subjected to centrifugation at 7000xg for 10 minutes. The serum was kept at -80 °C for later analysis.

Subretinal fluids were collected during surgical repair of the detached retina. The area around the drainage site was cleared of irrigation fluid and blood. The sclera was punctured by a 27 Gauge needle passed perpendicularly to the sclera where the retinal detachment was the most bullous and major choroidal vessels were avoided. The needle was advanced for about 0.6 to 0.8 mm deep to enter into the subretinal space. The globe was gently massaged to facilitate drainage of the subretinal fluid via the needle to a tuberculin syringe. The specimen was then centrifuged for 10 minutes at 7000 xg to remove the cellular components.

3. Extraction of lipid component

(a) Retina: Each sample was mixed with 200 μl deionized water and sonicated for 2 minutes. An equal volume of acetone was added to coagulate the proteins. The

sample was mixed with 800 μl hexane by vertex. After sonication and centrifugation, the upper supernatant was removed and evaporated to dryness in the lyophilizer. The hexane extraction was repeated twice and the lipid extract was combined and evaporated to dryness. The lipid residue was re-dissolved in 16% dioxane in hexane for HPLC analysis.

(b) serum or subretinal fluid: An aliquot of 0.2 ml of sample was mixed with an equal amount of absolute ethanol. Lipids in the samples were extracted by mixing vigorously with 0.8 ml of hexane for 3 min. The organic phase was separated from the aqueous phase by centrifugation at 2000 xg for 10 min. The aqueous phase was extracted in the same manner for two more times. The supernatant fractions were combined and evaporated to dryness in a Maxi Dry Lyo (Heto Lab. Equipment, Allerol, Demark). The residue was re-dissolved in 25 μl of dioxane-hexane (16:84) immediately before chromatographic analysis.

(c) liver: The liver tissue was cut into small pieces by a surgical blaze. About 10 to 20 mg sample was weighed and homogenized in 0.2 ml of deionized water by sonication in a Branson Sonifer-450 for 6 minutes. The homogenate was mixed with an equal amount of acetone and vortexed for two minutes followed by the addition of 0.8 ml hexane. The mixture was further sonicated and vortexed for 1 and 2 minutes respectively. The supernatant of the hexane extraction was removed after centrifugation at 5000 x g for 5 minutes. Hexane extraction was repeated twice and the extracts were recombined and evaporated to dryness in the lyophilizer.

4. Analytical methods

(a) Isocratic elution: A Nova-Pak silica column (3.9 x 300 mm) was equilibrated with 16 % dioxane in hexane and eluted at 2 ml/min. Pressure was maintained around 1220 to 1340 psi under silk on condition. The dried organic extract of each sample was then redissolved in 50 µl elution solvent and 40 µl of the sample was injected into the column. The eluant was monitored for 15 minutes after injection. The chromatogram was analysed at 450 and 327 nm.

(b) Gradient elution: The lipid extract was dissolved in 100 µl hexane and 50 µl was injected into the column. The samples were eluted in the gradient mode from 0% to 20 % dioxane within five minutes after sample injection. The concentration of dioxane was kept constant at 20 % dioxane after 5 minutes of gradient elution. The elution was monitored with a photo-diode array detector. The chromatogram was analysed at 450 and 327 nm.

III. RESULTS

A. Selection of chromatographic method for carotenoids analysis.

1. Effect of dioxane concentration on the retention time (T_r) of carotenoids: The separation of carotenoids on a silica column was examined by isocratic and by gradient elution.

(a) Gradient elution (Table 1): Gradient elutions using 5-25%, 0-16% and 0-20% dioxane in hexane were examined. When dioxane concentration was changed from 5 to 25% in the first 5 minute, ß-carotene and lycopene were barely separated [T_r = 1.43 ± 0.01 (n=2) and 1.55 ± 0.0058 (n=2)]. Lutein was eluted at 7.9 minute. When the gradient was changed to 0-16% dioxane in the first 5 minute, ß-carotene and lycopene were separated by 1.2 minute. The T_r of lutein was delayed to 14.83 minute. When dioxane concentration was changed from 0 % to 20 % at the first five minutes, similar separation of ß-carotene and lycopene was observed while lutein was recovered much earlier at 10.6 ± 0.1 (n=4) minute.

(b) Isocratic elution (Table 2): In our earlier studies, the 3.9x150 mm column was used. The concentrations of 15, 16, 25 and 30% of dioxane in hexane were used in isocratic elution. There was an inverse correlation between the T_r to the concentration of dioxane. The T_r of each carotenoid increased as the concentration of dioxane reduced. At a specified elution solvent, the order of carotenoid eluted from the column were β-carotene, astaxanthin-1, lutein, zeaxanthin and astaxanthin-2.

When the silica column was eluted by dioxane and hexane (25:75, V/V) at 1ml/min. The T_r of ß-carotene, astaxanthin, lutein and zeaxanthin were 1.39 ± 0.01

(n=10), 3.73 ± 0.24 (n=9), 4.17 ± 0.12 (n=7) and 4.54 (n=1) respectively. When the elution concentration of dioxane was changed from 25% to 30 %, the T_r of all carotenoids reduced. The changes in T_r of oxygenated carotenoids were much larger than carotenes.

When the ratio of dioxane to hexane was changed to 15: 85, the T_r of ß-carotene, lutein and zeaxanthin were delayed to 1.44 (n=1), 13.25 ± 0.27 (n=4) and 14.16 ± 0.081 (n=2) respectively. Under the same condition, the cis and trans isomer of astaxanthin could be separated. The T_r of the trans isomer (astaxanthin-1) and the cis isomer (astaxanthin-2) were 10.02 ± 0.02 and 12.97 minutes respectively (Table 2).

In order to give a better separation for the carotenoids, a long silica column (3.9 x 300mm) was evaluated. The samples were eluted from the column at a flow rate of 2 ml/min. When β-carotene, lycopene, cryptoxanthin, astaxanthin-1, astaxanthin -2, lutein, zeaxanthin were eluted with 16% dioxane in hexane at 2ml/min. Their T_r were 1.35 ± 0.02 (n=7), 1.38 (n=1), 3.32 ±0.01 (n=2), 9.83 ± 0.72 (n=2), 12.02 (n=1), 10.76 ± 0.17 (n=3) and 11.56 ± 0.85 (n=5)minutes respectively. The T_r of astaxanthin and lutein eluted at 1 ml/min with 25% dioxane were shown in Table 3.

2. Chromatograms of carotenoids and retinoids of a selected method for routine analysis: A long silica column (300 mm) eluted with 16 % dioxane in hexane was chosen for routine analysis. The pressure was between 1200 to 1300 psi at 2ml/min. Carotenoids eluted from the column was detected by their absorbance at 450 nm. Each peak in the 450 nm chromatogram was confirmed to be a carotenoid by its

characteristic absorption spectrum between 400 and 550 nm. For the study of retinoids, the eluant was analysed at 327 nm. The spectra of retinoids were examined between 300 and 400 nm. The typical chromatograms and spectral analysis of carotenoids and retinoids are described in the following:

(a) Carotenoids: Carotenoids have a unique absorption spectrum between 400-550nm. The chromatograms of the carotenoids standard were shown in figures 1a to 6a. The shapes of the spectra were shown in figure 1b-6b. The A_{max} and T_r of these standards were shown in table 4.

ß-carotene has the typical triple-peak absorption spectrum in the visible region from 400 to 500 nm (figure 1b). Besides the minor peaks below 360 nm, the spectrum of ß-carotene (figure 1b), cryptoxanthin (figure 3b), lutein (figure 5b) and zeaxanthin (figure 6b) are nearly the same. The A_{max} of each peak has a difference of 2 nm only (figure 1b, 3b, 5b and 6b). The spectrum of lycopene has a much sharper absorption peak than other carotenoids. The main absorption bands have a bathochromic shift (figure 2b). The absorption spectrum of lutein is very similar to that of ß-carotene except a mild spectral shift to short wavelength. It is also accompanied by a slight increase in crinkle of A_{max} (figure 5b). Astaxanthin-1 has a single absorption peak at 475 nm rather than three distinct peaks at the visible region (figure 4b). The spectrum is smooth with a small shoulder at 369 nm. The spectrum of astaxanthin-2, comparing with astaxanthin-1, has a 7 nm hypsochromic shift of the main absorption band. The presence of the absorption band at 369 nm denotes the cis-isomer of the astaxanthin (figure 4b)(Britton, 1995),

(b) Retinoids; Retinol and its metabolites are separable by the present HPLC method. The chromatogram was analysed at 320 nm to detect retinoids (figure 7a). Each retinoid has its characteristic $A_{max.}$. The absorption spectra of the retinoids were shown in figure 7b.

The A_{max} of each retinoid locates between 300 and 400 nm. Both retinol and retinol acetate have the absorption maximum at 327 nm and retinol has an additional A_{max} at 230 nm. However, the T_r of retinol acetate is 1.58 minute while the T_r of retinol is 3.05 minute. The T_r for retinal and retinoic acid are 1.87 and 2.64 minutes respectively (figure 7a). The A_{max} of retinal is 370 nm while the A_{max} of retinoic acid further shift to 350 nm.

B. Applications of the selected method to study carotenoid in human and rats.

1. Study in human serum and subretinal fluid.

(a) Serum: The concentrations of different carotenoids and retinol in the normal serum were displayed in table 5. At least 8 peaks were found in the 450 nm chromatogram of serum (figure 8a). All peaks had the typical A_{max} with the appearance of triple peaks of carotenoids between 400 and 500 nm (figure 8b, c). Only β-carotene, cryptoxanthin, lutein and zeaxanthin were recognised by their T_r and A_{max} (Table 4). Other carotenoids have not been identified yet. β-carotene had the highest concentration among the carotenoid found in the blood. Lutein was another major carotenoid. Only a trace amount of zeaxanthin was found in the blood and the concentration was too low to be documented by its absorption characteristics.

When the serum was analysed at 327 nm, the major peak (peak 5) located at 3.1 minute (figure 9a). The A_{max} and T_r matched with that of retinol (Table 4). The A_{max} of peak 1 to 4 indicated the presence of a mixture of unknown compounds. The possible occurrence of retinol ester, retinal and retinoic acid could not be identified by this method. The last peak has a spectrum of retinol and it is shown by heavy line (figure 9b).

(b) Subretinal fluid: When the subretinal fluid was analysed at 450nm, lutein was the major peak (figure 10a). The ß-carotene peak (peak 1) was present, yet its concentration was < 3% of that in serum (table 6). A small amount of zeaxanthin was found in the subretinal fluid. The SRF/serum ratio for zeaxanthin and lutein were similar. The absorption spectrum of ß-carotene (peak 1), lutein and zeaxanthin peak

(peak 7, 8) were shown by heavy lines in figure 9b. The spectrum was distorted by the base line noise due to the low concentration of carotenoids injected to the column. The result was sufficient to illustrate the absorption characteristic of ß-carotene and lutein. The concentration of zeaxanthin was too low to be identified by the absorption spectrum. The other compounds eluted near the location of ß-carotene had strong absorption at low wavelength UV light (<350nm). Yet negligible absorption in the visible light (450-550 nm) interfere with the absorption characteristic of carotenoids.

The chromatogram of subretinal fluid recorded at 327 nm showed a group of peaks with low T_r (< 3 min) (figure 11a). The last peak (T_r = 3.05 min) was clearly separated from the others and had the typical absorption spectrum of retinol (figure 11b, heavy line). The earlier peaks overlapped each other. The chromatograms and spectra of the subretinal fluid at 450 and 327 nm (figure 10-11a,b) were qualitatively similar to that of serum (figure 8-9a,b).

The average values of retinol, ß-carotene, cryptoxanthin, lutein and zeaxanthin in serum and subretinal fluid are shown in table 6. The concentration of retinol in the subretinal fluid is 30% of that in the serum. The concentration of lutein in subretinal fluid is 38.7 ng/ml, equivalent to 26 % of that in serum. In spite of the high amount of retinol and lutein in subretinal fluid, the concentration of ß-carotene and cryptoxanthin in subretinal fluid is very low (2.5 % and 2.8% of serum). The concentration of zeaxanthin in the subretinal fluid was much lower than that of β-carotene but the relative percentage was 22 % of that in the serum. There was no detectable lycopene in subretinal fluid.

2. Studies in rat's tissues

(a) Liver:

Normal diet (figure12): Normal liver had 2 peaks (T_r at 3.8 and 4.4 minutes) in the 450 nm chromatogram (figure 12a). Peak 1 had an A_{max} located at 205, 275 and 327 nm. Peak 2 had A_{max} at 205 and 275 nm (figure 12b). Both peaks had strong UV absorption below 250 nm and they did not have the absorption spectrum of carotenoids even after they were highly magnified in figure 12c.

Oil supplement (figure 13): When rats were fed with olive oil alone, five peaks were noted. The T_r were 1.95, 3.35, 4.19, 6.57 and 10.9 minutes respectively (figure 13a). Peak 3 had the highest absorption among the five peaks. Peak 1 had an undefined spectrum with its A_{max} at 210 nm. It also had one shoulder at 280 nm (figure 13d). Peak 2 had a very strong absorption at 327 nm. The spectrum of peak 3 had the A_{max} at 270 nm and two minor peaks at 325 and 410 nm (figure 13b). Under high magnification, peak 1 and 4 had barely detectable absorption between 400 and 500 nm (figure 13d). These absorbances seemed to be carotenoids.

Lutein, 1 mg/kg supplement (figure 14): The chromatogram of the liver tissue with lutein (1 mg/kg) (figure 14a) was very similar to the oil fed group (figure 13a). The absorbances of the small peak (peak 5) at the T_r of lutein were approximately the same in both oil-supplement and 1mg/kg groups. Peak 1 had the multiple peaks between 350 and 400 nm while peak 3 possessed a board peak at 400 nm (figure 14c). According to

the spectrum of each peak, only barely detectable carotenoids could be observed in peak 4 (figure 14d).

Lutein, 30 mg/kg supplement (figure 15): As lutein dosage was increased to 30 mg/kg, peak 4 and 5 in the liver were raised to the concentration that could be identified as the spectrum of carotenoids (Figure 15c). The T_r of peak 5 was identical to that of lutein (figure 15a) and this peak had the absorption spectrum of carotenoid (figure 15c). Peak 2 and 4a had the A_{max} (327 nm) of retinol (figure 15b). None of the others had the A_{max} of carotenoids.

Astaxanthin, 1 mg/kg supplement (figure 16): The chromatogram was similar to the one fed with lutein 1mg/kg (figure 16a). After very high magnification of the spectra, only peak 4 had an appearance of carotenoids (figure 16c).

Astaxanthin, 10 mg/kg supplement (figure 17): Five peaks could be observed when astaxanthin (10 mg/kg) was fed to the rats. T_r were 1.95, 3.35, 4.2, 6.58 and 10.92 minutes respectively (figure 17a). Peak 1 had three multiple A_{max} located at 350, 369 and 393 nm. The shape of the spectrum had the triple peak appearance of carotenoids in the visible spectrum (figure 17c) although the wavelength was shifted toward the UV side. Peak 2 had strong A_{max} at 327 nm and the curve came down at 430 nm and flattened out onward. Peak 3 had strong absorption at 270 nm and deflections occurred at 323 and 400 nm. Peak 4 had strong absorption at 205 nm and flattened off to 300 nm. (figure 17b, c).

Astaxanthin, 30 mg/kg supplement (figure 18): High dose astaxanthin raised the peak height of peak 1, 2 and 4 in the 450 nm chromatogram (figure 18a). The spectrum of each peak was very similar to that of 10 mg/kg group (figure 17). Peak 4 showed a barely detectable absorbance between 400 and 500 nm (figure 18c). T_r of Peak 5 was corresponding to that of lutein. However, the spectrum was too weak to be identified as carotenoid.

(b) Serum

Normal diet (figure 19): When the animals were fed with normal diet alone, two peaks were noted with T_r of 1.44 and 1.73 minutes (figure 19a). Peak 1 had A_{max} at 210, 270, 325 nm (Figure 19b). Upon magnification, triple peak of carotenoid from 370 to 480 nm could be seen in peak 1. Although the triple peak had visible colour, the A_{max} shifted to the shorter wavelength (figure 19c). Peak 2 had strong absorption at low UV region and 295 nm. Little absorbance was observed at the visible wavelength.

Oil supplement (figure 20): When olive oil was fed to the rats, 4 small peaks were observed in the 450 nm chromatogram of the serum (figure 20a). Their T_r were 1.45, 1.72, 9.2 and 11.3 minutes respectively. Peak 1 had very strong absorption in the low UV region and the patterns were similar to the untreated group. Peak 1 level off from 275 to 400 nm (figure 20b). Peak 2 had A_{max} at 290 and 350 nm with a new A_{max} introduced at 250 nm. T_r of peak 3 and 4 corresponded to the T_r of astaxanthin-1 and

astaxanthin-2. However, no carotenoids absorption characteristics could be detected for all four peaks in spite of high magnification (figure 20c).

Lutein, 1 mg/kg supplement (figure 21): In lutein-fed group, the T_r of the 450 nm peak were 1.4, 1.7, 5.9 and 11.2 minutes (Figure 21a). Peak 1 and 2 had high absorbance below 350 nm and no absorption characteristics of carotenoids. Peak 1 had an A_{max} at 275 nm. The shape of spectrum in peak 2 was similar to that in oil-fed group except the absence of A_{max} at 250 nm. Peak 3 and 4 showed only barely detectable absorbance between 400 and 550 nm (figure 21c). The T_r of the fourth peak matched with the standard of lutein, however, one could not identify the presence of carotenoid according to the spectrum.

Lutein, 10 mg/kg supplement (figure 22): When 10 mg/kg lutein was fed to the rats, 4 peaks were found in the 450 nm chromatogram (figure 22a and 22b). T_r were 1.57, 1.96, 2.55 and 10.75 minutes respectively. Peak 1, 2 and 3 had an A_{max} at 432 nm (figure 22c, d). T_r of lutein and peak 4 were the same. However, the absorption was so weak that carotenoids could not be identified by the spectral analysis.

Astaxanthin, 1 mg/kg supplement (figure 23): At a low dose (1 mg/kg) of astaxanthin, the chromatogram of the serum (figure 23a) had four peaks detected at 450 nm. T_r of the 4 peaks were 1.4, 1.7, 9.2 and 11.8 minutes respectively. Peak 1 and 2 had high absorption below 300 nm and negligible absorption in the visible absorption band (figure 23c). The spectrum of peak 2 was similar to the one of oil-fed group. Spectra of

peak 3 and 4 were shown to have a very low carotenoid absorbance under very high magnification (figure 23d). They matched with the T_r of astaxanthin-1 and astaxanthin-2.

Astaxanthin, 10 mg/kg supplement (figure 24): The chromatogram of the serum in a high dose (10 mg/kg) of astaxanthin showed 4 peaks at 450 nm (figure 24a). There was a marked rise in peak 1 and 2. T_r of the peaks in this group were 1.57, 1.95, 2.47 and 10.66 minutes. The spectra of peak 1 and 2 appeared as a board peak at 432 nm (figure 24d) like astaxanthin. However, their T_r were quite different from astaxanthin. These peaks with A_{max} at 432 nm were present only in the group fed with 10 mg/kg carotenoids. Although T_r of peak 4 matched with the standard lutein, the absorbance was too low that it could not be identified by its spectrum as carotenoid.

(c) Retina

Normal diet (figure 25): The retina of normal untreated rats had two peaks when the chromatogram was processed at 450 nm (figure 25a). The T_r were 1.4 and 1.5 minutes respectively. Peak 1 had A_{max} at 210, 260 and 280 nm. Peak 2 had Amax at 210, 280 and 350 nm. Both of them consisted of mixtures of undefined absorption spectrum (figure 25b). No carotenoid was detected when the spectrum was highly magnified (figure 25c).

Oil supplement (figure 26): When olive oil was fed to the rats, the 450 nm chromatogram of the extracted retina revealed four peaks (figure 26a). Their T_r were

1.45, 1.7, 1.93 and 9.89 minutes. Peak 1 had a very strong UV absorption with several A_{max} at 210, 275 and 320 nm (figure 26b). Peak 2 had A_{max} at 210, 275 and 350 nm. Peak 3 had A_{max} at 210, 280 and 384 nm. The strong absorbance of peak 1 to 3 override the absorption from 400 to 470 nm. Although T_r of peak 4 closed to that of lutein, the spectrum had very little absorption between 400 to 550 nm (figure 26c).

Lutein, 1 mg/kg supplement (figure 27): Five small peaks were recorded in the 450 nm of lutein-fed retina (figure 27a) with T_r 1.42, 1.67, 1.89, 7.1 and 7.7 minutes. All spectra of each peak had several A_{max} at the UV absorption band (Figure 27b). Peak 1 and 2 had maximum absorption below 300 nm with no absorbance characteristic of carotenoids (figure 27c). Peak 1 had one A_{max} at 210 nm. Peak 2 had A_{max} at 210, 240, 280 and 350 nm. Peak 3 had the same A_{max} as in oil-fed group. Peak 4 and 5 had absorption between 400 and 550 nm region. However, their T_r did not match with any of our standard carotenoids.

Lutein, 10 mg/kg supplement (figure 28): Four peaks were recorded in the chromatogram of lutein-fed group (figure 28a). Their T_r were 1.45, 1.66, 1.92 and 6.48 minutes. Peak 1 had high absorption below 300 nm with a shoulder at 323 nm. Peak 2 had A_{max} at 210, 270 and 350 nm(figure 28b). Peak 3 had A_{max} at 256 and 384 nm. The absorbance in peak 4 was so low that carotenoids could not be confirmed by its spectrum (figure 28c).

Astaxanthin, 1 mg/kg supplement (figure 29): Four peaks were shown in the chromatogram of retina in the astaxanthin-fed group (figure 29a). The T_r of the peaks were 1.43, 1.69, 1.92 and 9.79 respectively. The absorbance of peak 1 to 3 were nearly the same as the tissue fed with 10mg/kg lutein. Peak 4 had absorbance between 450 and 550 nm only in highly magnified spectrum. The absorption was so low that its identity to astaxanthin is questionable.

Astaxanthin, 10 mg/kg supplement (figure 30): When 10mg/kg astaxanthin was fed to the rats, three peaks with T_r 1.4, 1.7 and 1.9 minutes were seen in the chromatogram of the retina (figure 30a). The A_{max} of peak 1 located at 323 nm. Peak 2 had A_{max} at 350 nm while Peak 3 had A_{max} at 266 and 388 nm. No carotenoid was detected.

C. Influence of dietary carotenoids on retinol concentration in rats

1. Serum (Table 7): The concentration of retinol in normal serum of rat varied between 200 and 346 ng/ml (SD = 73 ng/ml, n=5). When the rats were fed with oil alone, the concentration of the retinol increased significantly (601 ± 165 ng/ml, n=11, $P<0.0001$). However, the administration of lutein or astaxanthin (30 mg/kg) to rats did not increase the retinol concentration further.

2. Retina (Table 8): The variations of retinol in the retina were large. Oil administration to rats did not affect retinol in the retina significantly. Feeding astaxanthin (10 mg/kg) reduced the retinol in the retina ($P<0.05$). However, high dosage of lutein, 30 mg/kg, increased retinol concentration in the retina ($P=0.0014$) comparing to the oil-fed group.

IV. DISCUSSION

A. Chromatographic analysis of carotenoids and retinoids.

Retinoids and carotenoids include a large family of structurally related compounds. They share a very similar hydrocarbon backbone with conjugated double bonds. They differ from each other in the size of the carbon chain and the number as well as the position of oxygen functional groups. The oxygen functional group is the major determinant of their polarity. The present results demonstrate the feasibility of separating these compounds according to their polarity. The nonpolar carotenes have low T_r. The oxygen functional groups of lutein, zeaxanthin and astaxanthin increase their T_r. The resolution of these compounds by HPLC is dependent on their polarity and the polarity of the mobile phase. Variation in the concentration of dioxane and hexane are used to adjust the polarity of the mobile phase. After a series of investigation on the T_r of different standards using a gradient elution or isocratic elution, an isocratic elution by 16% dioxane in hexane was selected for routine analysis.

Gradient elution has the flexibility in separating multiple compounds in one single chromatogram. The resolution is dependent on the slope of the gradient. The reproducibility is dependent on the stability of the solvent mixing system. Instrumental error in the solvent delivery system often contribute to analytical error. It is much easier to control the isocratic elution than a gradient elution. In this study, the gradient elution was used to select the optimal concentration of dioxane for isocratic elution.

The present data showed that an isocratic elution by 16% dioxane is the optimal condition for the analysis of retinol, ß-carotene, lutein, and astaxanthin in rat serum and

tissues. This method enables the separation of retinol, retinoic acid, retinol acetate and retinal. However, only retinol can be identified when lipid extracts of serum, subretinal fluid, retina or liver were analyzed. The other retinoids cannot be resolved with other unidentified lipids. Therefore, among retinoids only retinol is considered in this study.

The present method provides a fairly good separation of all carotenoids concerned in this study. The limitation is the separation of lycopene and ß-carotene. Fortunately, lycopene is not present in rat serum and tissues, nor in human subretinal fluid and retina. Lycopene and ß-carotene can be separated by reducing dioxane concentration to 1 %. In situation where separation of ß-carotene and lycopene was necessary, a gradient elution beginning with increasing dioxane from 0-20 % in 10 minute should be used.

B. Carotenoid study in human and rats

1. Carotenoids in human tissues

(a) Serum: The chromatographic system selected for our study produced data consistent with current knowledge of carotenoids. Table 9 showed that β-carotene is the major carotenoid in the human serum. However, lycopene is not detected in the serum in our normal subjects. Giuliano et al (1992) reported the rapid degradation of lycopene during the specimen preparation procedure. Alternatively, lack of intake of lycopene in Asian diet is another possible reason for the absence of lycopene in the serum. Lycopene is found mainly in tomato products. Dietary intake of Asian do not contain a high amount of tomato product on a regular basis. If the diet is lack of tomato product,

lycopene may not be detectable in the blood. Others (Krinsky, 1990; Handelman, 1991) reported that the ratio of zeaxanthin to lutein in the blood is about 10 to 25 %. The concentration of zeaxanthin in our sample was about 10 % of lutein. Retinol concentration of normal human is also similar to published values (Bowen et al 1993; Stacewicz-sapunntzakis, 1993).

(b) Subretinal fluid: The chromatographic procedure used in this study is a simple and sensitive method to analyse retinol and carotenoids concentrations in the subretinal fluid. Since the amount of ß-carotene and lycopene are very small in the subretinal fluid, the isocratic elution with 16% dioxane is adequate for the present study. A gradient of increasing dioxane from 0 to 20% has been used to demonstrate the separation of ß-carotene and lycopene. No lycopene was observed in the subretinal fluid (the negative result is not shown).

The high concentration of lutein in subretinal fluids confirmed previous observation that lutein is the major carotenoids in human retina (Bone, 1985; Handelman, 1988). It is difficult to obtain the subretinal fluid without a small amount of blood contamination. Assuming that there is no ß-carotene in the subretinal fluid, the small amount (<4%) of ß-carotene and cryptoxanthin in subretinal fluid can be used to estimate the maximal blood contamination. Since the amounts of retinol, lutein and zeaxanthin in the subretinal fluid are 32 %, 26 % and 22 % of those in the serum respectively, we conclude that the occurrence of retinol, lutein and zeaxanthin are not resulting from blood contamination during sampling of specimens. The marked differences between the chromatograms of serum and subretinal fluid indicate the origin of these compounds from the degenerating retina rather than from the blood.

Bone (1993) proposed the conversion of meso zeaxanthin, a non-dietary carotenoid, from lutein in the retina. Their data indicate that zeaxanthin is derived from lutein in the retina. On the other hand, most of our patients suffering from mid-peripheral or peripheral retinal detachment. Lutein is the major carotenoids found in the mid-peripheral retina while zeaxanthin has the highest concentration in the macula region (Handelman, 1988; Bone, 1997). When the mid-peripheral retina detached, a relatively large amount of lutein would be released from the detached retina.

Both retinol and carotenoids are highly insoluble in an aqueous medium. Their occurrence in the subretinal fluid must be maintained in suspension by binding to proteins. The occurrence of retinol binding protein in the subretinal fluid has been reported by Bridges et al (1986). It is likely that carotenoids in subretinal fluid are also bound to proteins.

The present data show that considerable amounts of retinol and carotenoids are present in the subretinal space. If retinol and carotenoids are lost from the retina following retinal detachment, the re-attached retina is deficient in these important nutrients. The amount of these compounds remaining in the retina, and the ability to replenish them could be important factors affecting the surgical outcome.

2. Dietary supplement of carotenoids to rats

(a) Choice of animal and types of carotenoids: Photic injury to rat's retina has been studied extensively in our laboratory and by others (Penn, 1987; Lee et al 1990; Penn and Anderson, 1991). The protective effect of carotenoids in Lewis rats has been reported (Lam 1996). Therefore rat is chosen for the present study.

Oil was administered to the animals as a vehicle because of the hydrophobic properties of carotenoids. Olive oil had been shown to increase the bioavailiability of the carotenoids during absorption (Erdman et al, 1993; Solomons et al, 1993). We observed the appearance of peak 1 in the 450 nm chromatogram after feeding oil. The multiple peaks may be originating from the metabolic products of oil.

Lutein is one of the major carotenoids in the human retina. We have demonstrated that a considerable amount of lutein was present in the human subretinal fluid. If one attempt to apply the animal model to study human diseases, lutein would be a good choice. Thus we fed the rats with lutein as one part of this study.

Astaxanthin was selected in parallel with lutein feeding. *In vitro* study demonstrated the greatest antioxidant effect of astaxanthin among carotenoids (Miki, 1991). Moreover, protective effect against photic injury by astaxanthin had been reported in the rat model (Lam & Tso, 1996). Consequently, assessment of these two pigments has potential application for human retinal diseases. These two pigments are now available for poultry use. They are commercially accessible.

(b) Carotenoids uptake into rats' tissues: The chromatographic method was used to analyze carotenoids in liver, serum and retina of rats after feeding oil, with or without carotenoid supplement. The possible occurrence of carotenoids was examined first in the 450nm chromatogram. Each peak was re-examined by its spectral characteristic. Although many peaks were observed at 450nm, most of them do not have the spectral characteristic of carotenoids. When a high amount of lutein (30mg/Kg) was fed to rats, a small amount of carotenoid was noticeable in T_r of lutein in the 450 nm chromatogram of the liver extract (figure 15a, peak 4 and 5). Its concentration was

sufficient to show absorption spectrum of carotenoids (figure 15c). Only barely detectable lutein was found in the liver extract when 1 or 10 mg/kg of lutein was fed. The amount of carotenoid in the astaxanthin fed group was too low to be confirmed by the absorption spectrum (see figure 16-18). When the serum (figure 21-24) and retina (figure 27-30) were analysed, lutein and astaxanthin could not be confirmed by their absorption spectrum in the 450 nm chromatogram.

Carotenoids are believed to have protective effect against free radicals damage. The absence of carotenoid in rat's model explains their susceptibility to photic injury. Other have demonstrated the protective effect against photic injury by oral administration of lutein and astaxanthin to rats. These carotenoids must be very potent since the present data showed that only a trace amount of orally administrated carotenoid entered rat's tissue.

Although feeding lutein or astaxanthin did show the appearance of the respective carotenoid in some specimen of the blood or tissues, the concentration was in the lowest limit of our analytical method. This concentration was <1% of the values observed in human serum.

Because of the hydrophobicity of carotenoids, a carrier protein is required to keep them in the blood circulation, and transport into the retina. Carotenoid binding protein in the human retina has been reported recently by others (Bernstein et al 1997; Crabtree, 1997). No study has been reported on the carotenoids binding protein in serum. The extremely low concentration of carotenoids in the rats serum after an excessive dosage of carotenoid could be resulting from the absence of the carrier protein for carotenoid in rats.

(c) Effect of dietary carotenoids on retinol concentration in rat tissues: Supplementation of oil significantly increased the concentration of retinol in the serum. Addition of carotenoid in oil did not raise the retinol concentration further. Thus, the change in retinol was affected by the oil rather than carotenoids. In our preliminary study, retinol or carotenoids was not found in the olive oil. Therefore, retinol is not originating from oil. It is possible that oil promotes the absorption of dietary retinol.

In the retina, the influence to retinol was different from that of serum. Oil or astaxanthin (10 mg/kg) reduced retinol while lutein markedly elevated the concentration of retinol in the retina. Vitamin A had been proposed to have antioxidant effect against lipid peroxidation (Livrea and Packer 1994). High dosage of lutein may have preserved vitamin A in the retina.

V. CONCLUSION

Elution of retinoids and carotenoids from a silica column by 16% dioxane in hexane has been shown to be quite suitable for the present study of retinol and carotenoids in human and rat tissues.

Studies on the carotenoids in human serum and subretinal fluid confirmed the specificity of lutein as the major carotenoid in human retina. Although abundant amounts of several carotenoids are present in human serum, only a considerable amount of lutein is present in the subretinal fluid. The data support the previous observation of lutein as the major carotenoid in the retina (Bone 1985, 1988, 1997). Although zeaxanthin is the major carotenoid in the macular, the net amount of zeaxanthin is too small to appear as a major carotenoid in the subretinal fluid. The presence of retinol and lutein in the subretinal fluid indicates their possible clinical significance as markers to estimate the extent of tissue damage after retinal detachment.

Carotenoids are believed to have protective effect against free radical damage. The absence of carotenoids in rat's retina explains their susceptibility to light damage. Others have demonstrated the protective effect of astaxanthin and lutein against photic injury to rat's retina. The potency of the carotenoid must be very high because only a trace amount of carotenoid was observed in some rats after a high dosage of lutein or astaxanthin was fed to them.

There is a possibility that the mega dose of orally administrated carotenoids can spare the catabolic reactions of retinol, other physiological antioxidants, or reduce the free radical level in the blood circulation. Oral administration of a high dose of lutein

did increase retinol concentration in the retina but not in the serum. Future investigations should be directed to the influence of carotenoids on the concentration of other physiological antioxidants such as ascorbic acid, vitamin E, glutathione as well as the concentration of free radicals in the blood circulation.

This study illustrates a very important difference in carotenoid metabolism between rats and human. Human serum contains multiple carotenoids in amount easily detectable. Normal rats do not have detectable amount of carotenoids. In spite of orally administration of a high dose of carotenoid, only a trace amount was observed in the serum and tissues in rats.

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Table 1.

The retention time (min) of different carotenoids in several gradient elution conditions using the long column.

	n	Gradient 1	n	Gradient 2	n	Gradient 3
β-carotene	5	1.86±0.10	1	2.45	2	1.43±0.01
Lycopene	4	2.25±0.21	1	3.63	3	1.55±0.01
Lutein	4	10.60±0.10	1	14.83	2	7.96±0.03
Zeaxanthin	1	10.91	-	-	-	-

Gradient 1 : 0% →20 % dioxane in the first five minutes after injection.

Gradient 2 : 0% →16 % dioxane in the first five minutes after injection.

Gradient 3 : 5% →25 % dioxane in the first five minutes after injection.

Table 2.
The retention time (min) of different carotenoids in several isocratic conditions using the short silica column.

	n	15% dioxane, 1ml/min	n	25% dioxane, 1ml/min	n	30% dioxane, 1ml/min
β-carotene	1	1.44	10	1.39±0.01	-	-
Astaxanthin-1	2	10.02±0.02	9	3.73±0.24	4	3.23±0.15
Astaxanthin-2	1	12.97	-	cannot separate	2	3.87±0.14
Lutein	4	13.25±0.27	7	4.17±0.12	1	5.73
Zeaxanthin	2	14.16±0.08	1	4.54	-	-

Table 3.
The retention time (min) of different carotenoids in several isocratic conditions using the long column.

	n	16% dioxane, 2ml/min	n	25% dioxane, 1ml/min
β-carotene	7	1.35 ± 0.02	-	-
Lycopene	1	1.38	-	-
Cryptoxanthin	2	3.32 ± 0.01	-	-
Astaxanthin-1	2	9.83 ± 0.72	1	8.21
Astaxanthin-2	1	12.02	1	9.25
Lutein	3	10.76 ± 0.17	1	8.57
Zeaxanthin	5	11.56±0.85	-	-

Table 4.
Retention time (min) and absorption maximum (nm) of retinoid and carotenoids standard in the selected procedure. (See figure 1 to 7)

	Tr (minute)	A_{max} (nm)
Retinol acetate	1.6	327
All trans retinal	1.9	370
All trans retinoic	2.6	350
All trans retinol	3.1	327
ß-carotene	1.3	425, 451, 480
Lycopene	1.4	448, 471, 500
Cryptoxanthin	3.3	425, 451, 480
Astaxanthin-1	9.8	475
Lutein	10.7	422, 446, 475
Zeaxanthin	11.5	427, 453, 481
Astaxanthin-2	12.0	369, 468

Table 5.
The concentration (mean ± SD) of carotenoids in normal human serum

		ng/ml	nmol/ml
	n	Mean ± SD	Mean ± SD
β-carotene	13	259 ± 133	0.48 ± 0.25
Cryptoxanthin	13	209 ± 105	0.37 ± 0.19
Lutein	13	176 ± 60	0.31 ± 0.11
Zeaxanthin	13	4 ± 2	0.01 ± 0.00
Retinol	13	718 ± 24	2.51 ± 0.83

Table 6.
The concentrations (mean ±SD) of retinol and carotenoids in patients' serum and subretinal fluid

	n	Serum, ng/ml	Subretinal fluid, ng/ml	SRF/serum ratio %
ß-carotene	6	206 ± 47	5 ± 2	2.5%
Cryptoxanthin	6	180 ± 86	5 ± 2	2.8%
Lutein	6	147 ± 34	39 ± 25	26%
Zeaxanthin	6	9 ± 3	2 ± 3	22 %
Retinol	6	668 ± 155	215 ± 120	32%

Table 7.
The concentration of retinol in the serum of rats.

Group No.	Description	n	retinol per ml serum (ng/ml)
Group 1	Normal diet	5	273 ± 73
Group 2	Oil supplement	11	601 ± 165
Group 3	Lutein (30mg/kg) supplement	4	432 ± 82
Group 4	Astaxanthin (30mg/kg) supplement	14	505 ± 160

T-test

Group	P value
1 Vs 2	0.0001
1 Vs 3	0.0225
1 Vs 4	0.0006
2 Vs 3	0.0242
2 Vs 4	0.1572
3 Vs 4	0.2496

Table 8.
The concentration of retinol in the retina of rats.

	Description	n	weight of retinol (ng) per retina Mean ± SD	weight of retinol per gram retina (μg/g)Mean ± SD
Group 1	Normal diet	10	101.1 ± 52.0	7.3 ± 2.7
Group 2	+ olive oil	34	89.9 ± 49.3	5.2 ± 3.5
Group 3	+astaxanthin (10mg/kg)	30	75.6 ± 27.8	4.5 ± 2.0
Group 4	+lutein (30mg/kg)	12	136.4 ± 36.0	10.8 ± 4.6

Statistical test
Unpaired t-test

Group	P value (comparing wt of retinol)	P value (comparing wt of retinol per gram retina)
1 Vs 2	0.55580	0.06501
1 Vs 3	0.16713	0.01221
1 Vs 4	0.08873	0.03809
2 Vs 3	0.15182	0.33412
2 Vs 4	0.00181	0.00143
3 Vs 4	0.0000708	0.00054

Table 9.
The concentrations of carotenoids and retinoids in human serum

	N=48, women, *1	N=11 (4 man 7 women), *2	N=160, Caucasians, *3
β-carotene	207 ± 124	462 ± 318	190 ± 136
α-carotene	52 ± 57	165 ± 110	48 ± 37
Cryptoxanthin	136 ± 85	-	95 ± 72 (β-cryptoxanthin)
Lycopene	254 ± 176	481 ± 193	293 ± 163
Lutein	196 ± 69	-	115 ± 58
Retinol	434 ± 107	-	643 ± 175
Retinyl palmitate	-	-	73 ± 143
Zeaxanthin	-	-	30 ± 17

Mean ± SD in ng/ml
*1. P.E. Bowen 1993 (origin value converted from μg/dL).
*2. A. Carughi 1993.
*3. M. Stacewicz-sapunntzakis, Beaver Dam Eye Study (BDES) (origin value converted from μg/dL).

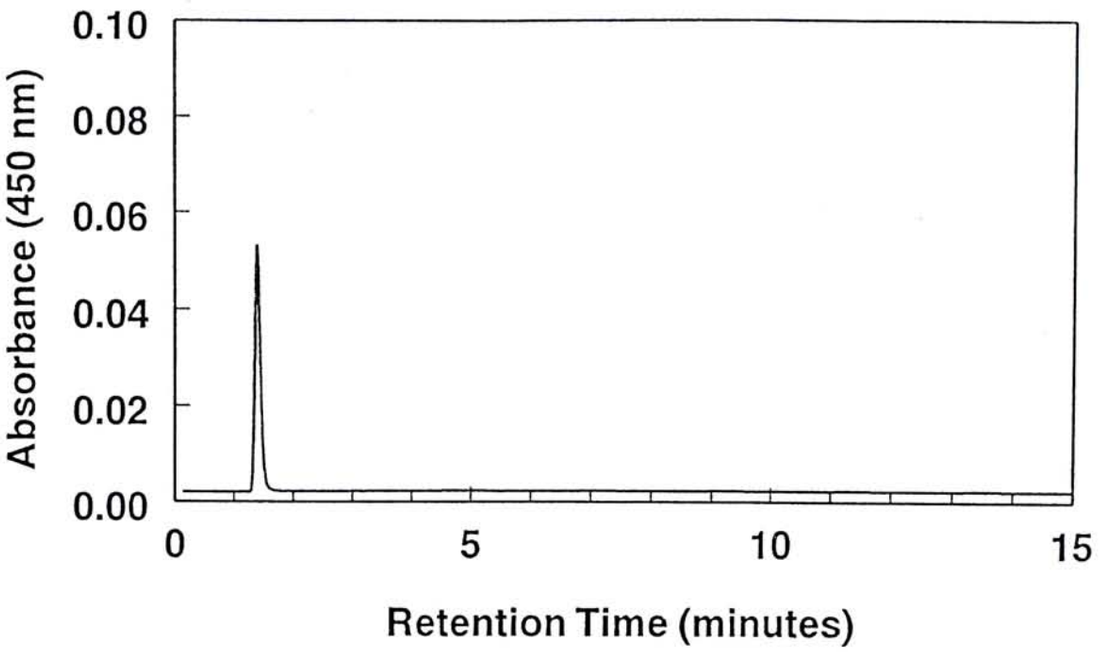

Figure 1a. Chromatogram of β-carotene standard at 450 nm.

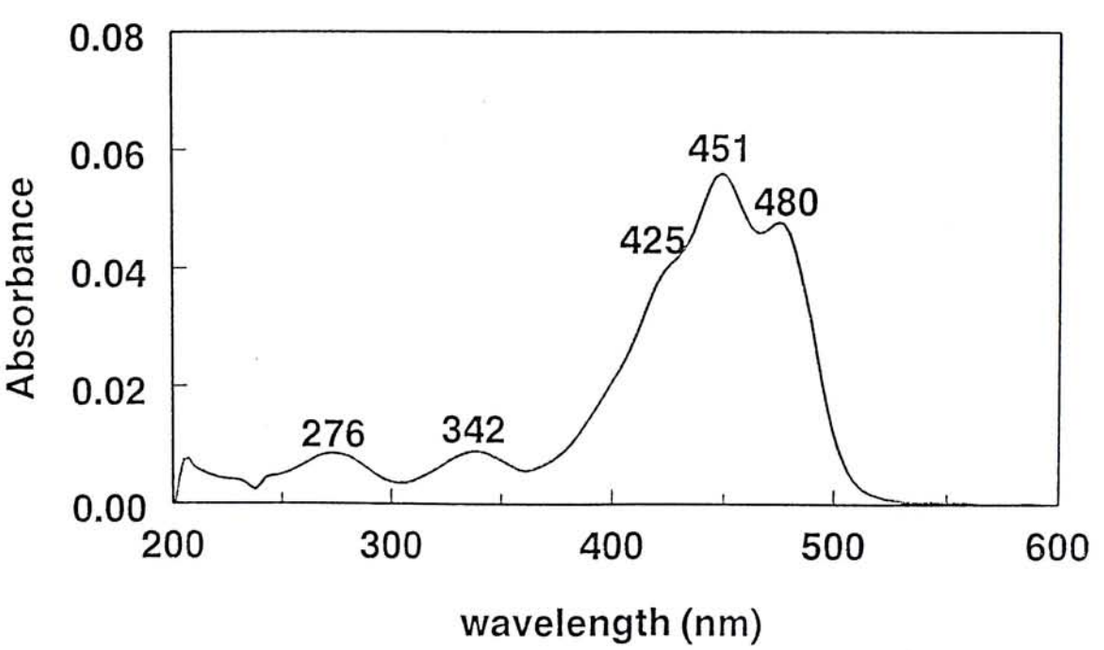

Figure 1b. Absorption spectrum of β-carotene

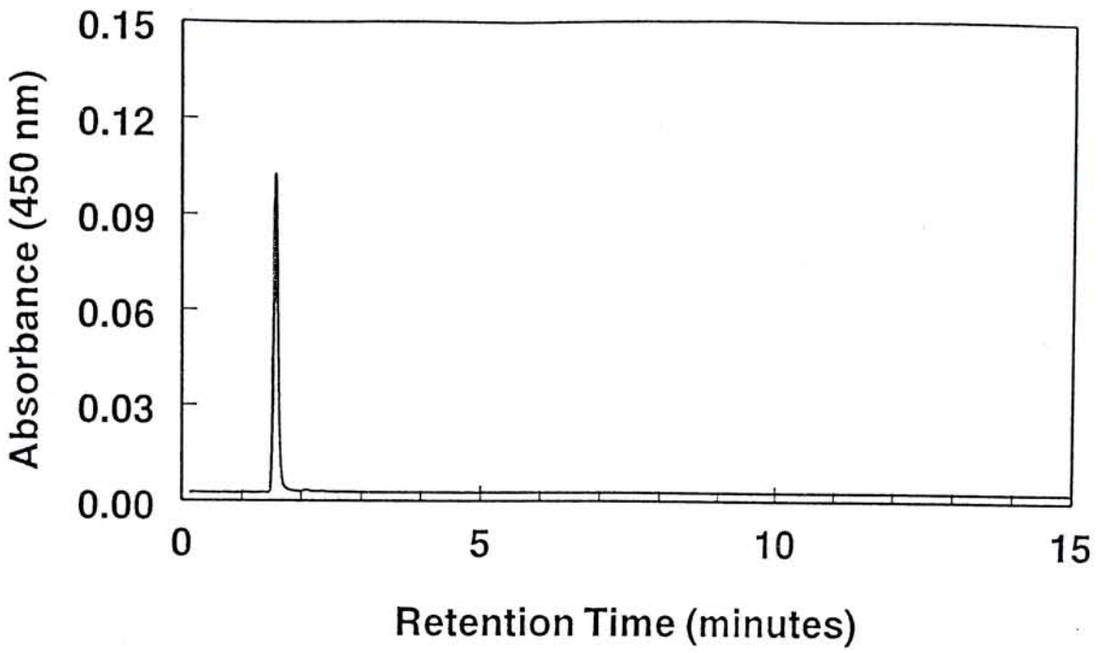

Figure 2a. Chromatogram of lycopene standard at 450 nm.

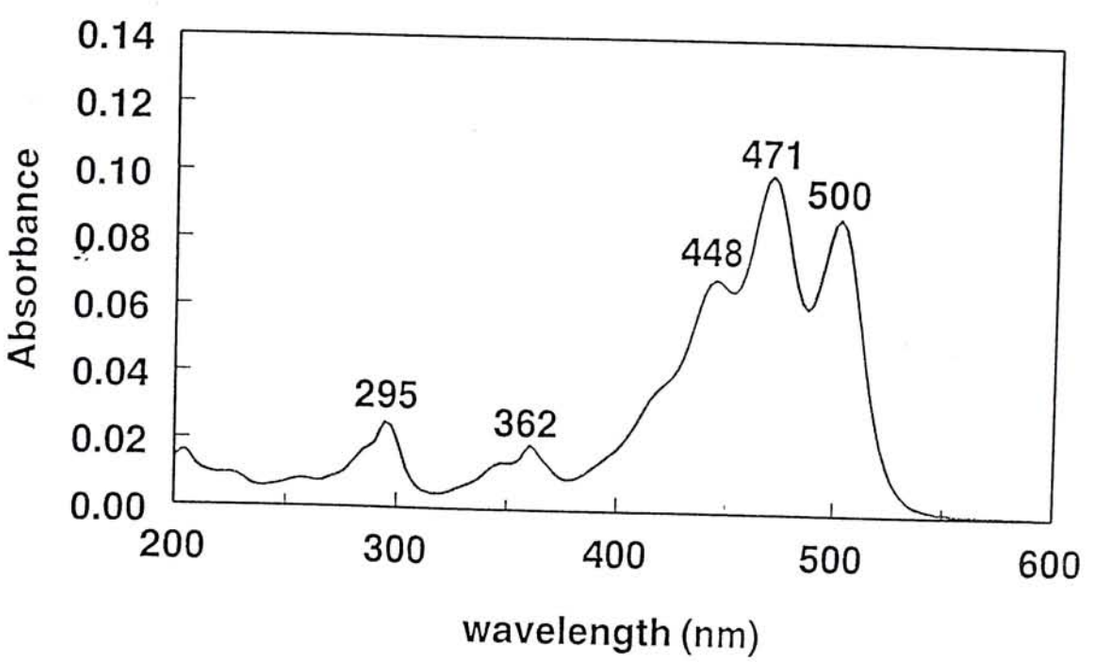

Figure 2b. Absorption spectrum of lycopene.

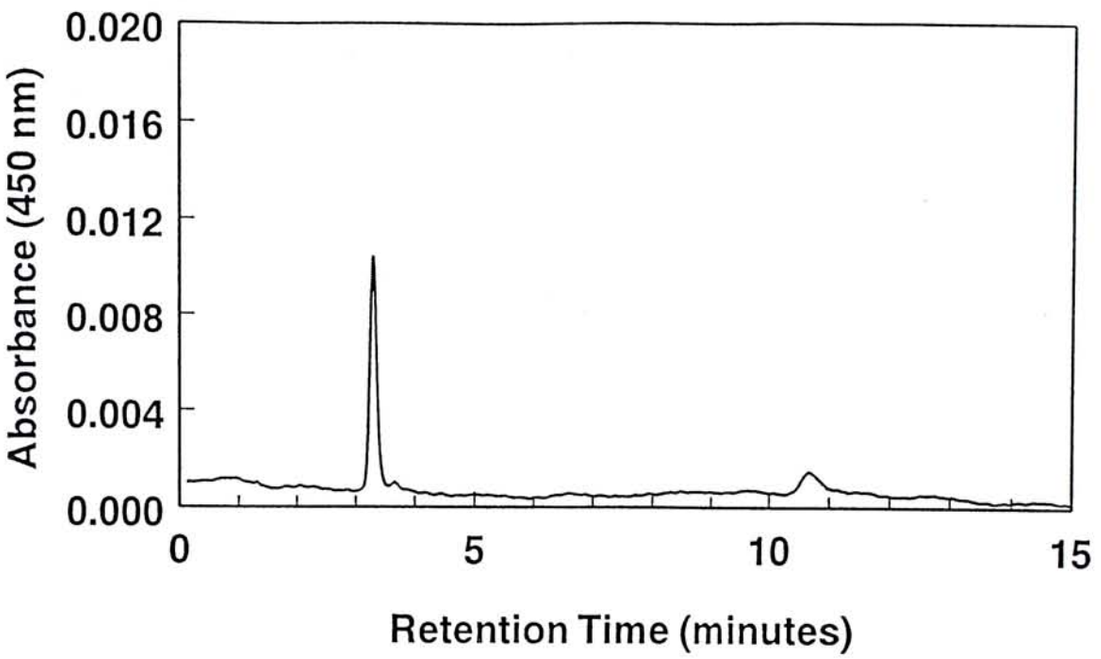

Figure 3a. Chromatogram of cryptoxanthin standard at 450 nm.

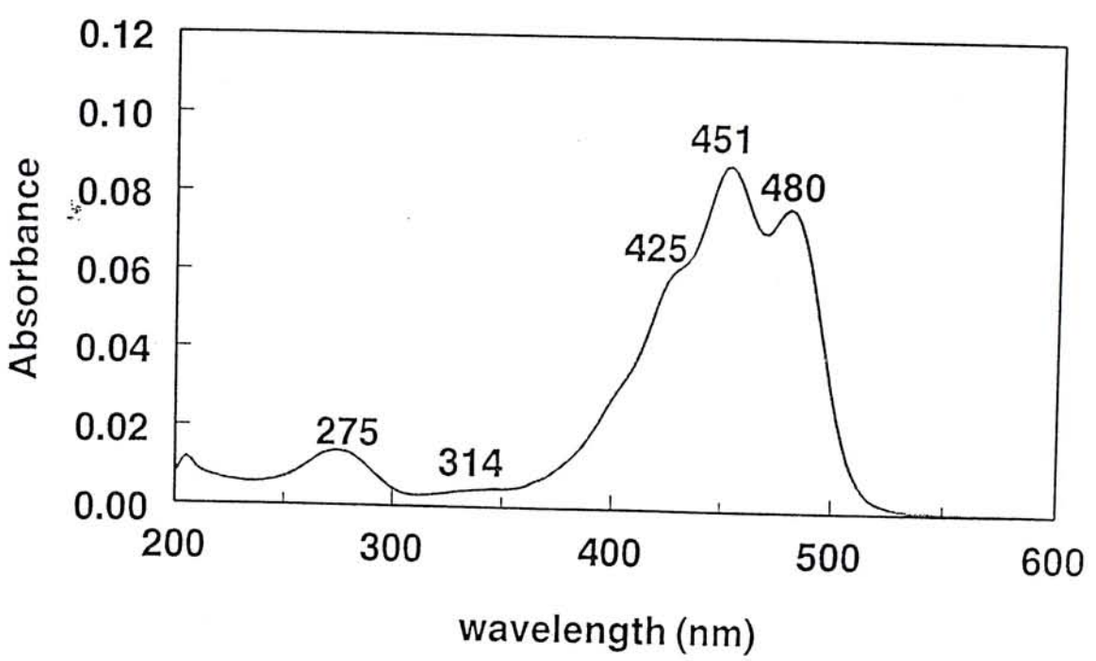

Figure 3b. Absorption spectrum of cryptoxanthin.

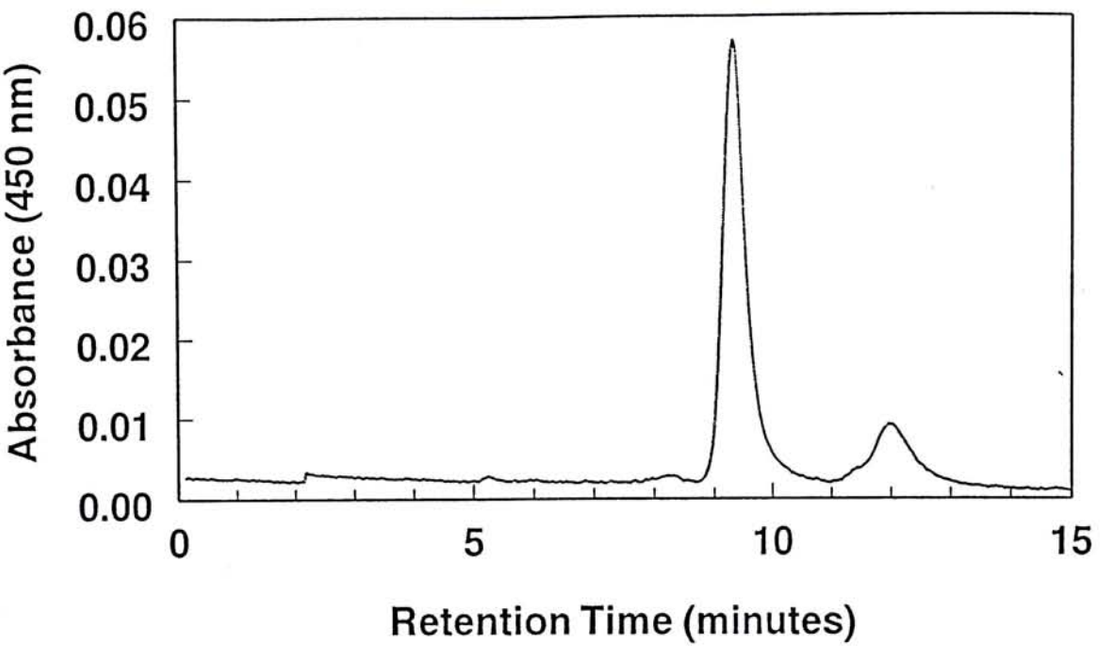

Figure 4a. Chromatogram of astaxanthin standard at 450 nm.

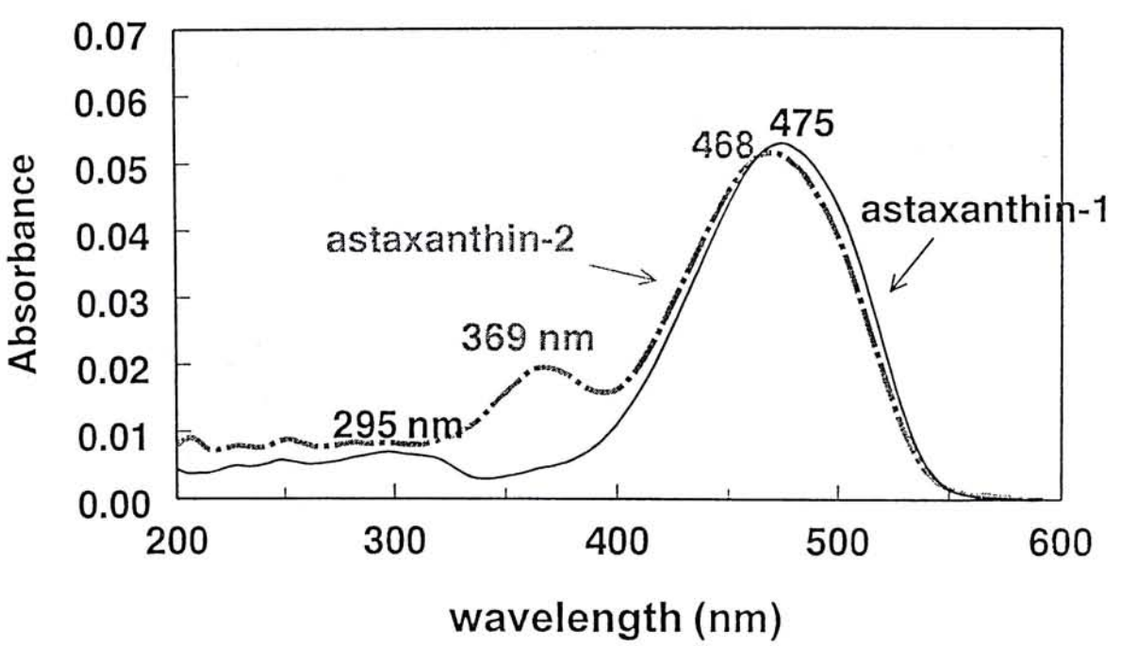

Figure 4b. Absorption spectra of astaxanthin-1, -2.

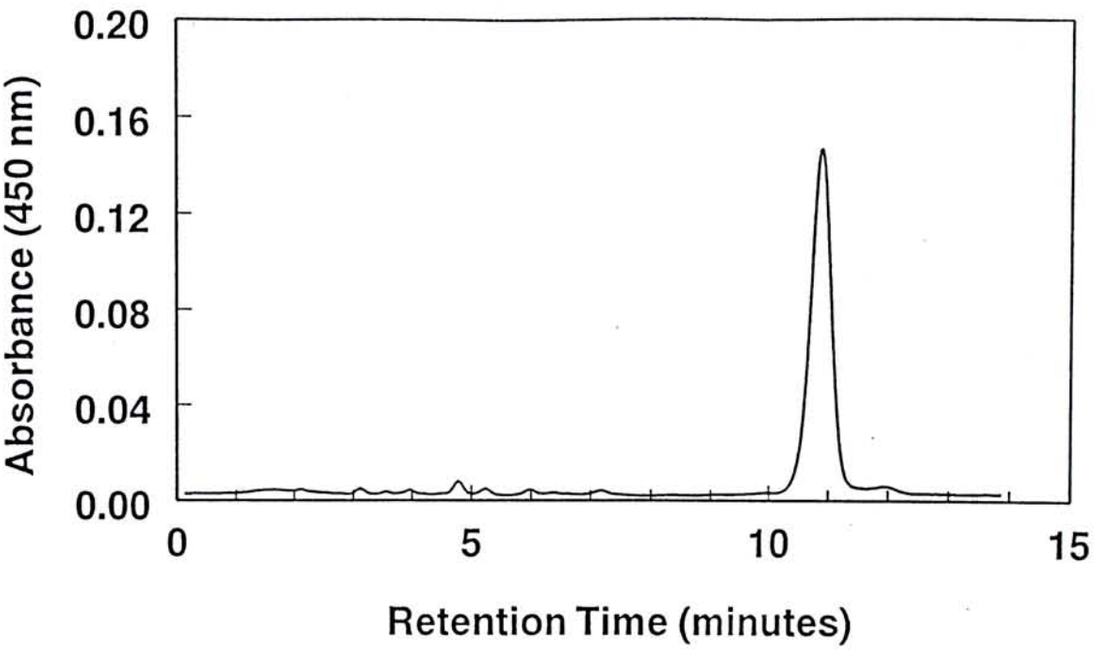

Figure 5a. Chromatogram of lutein standard at 450 nm.

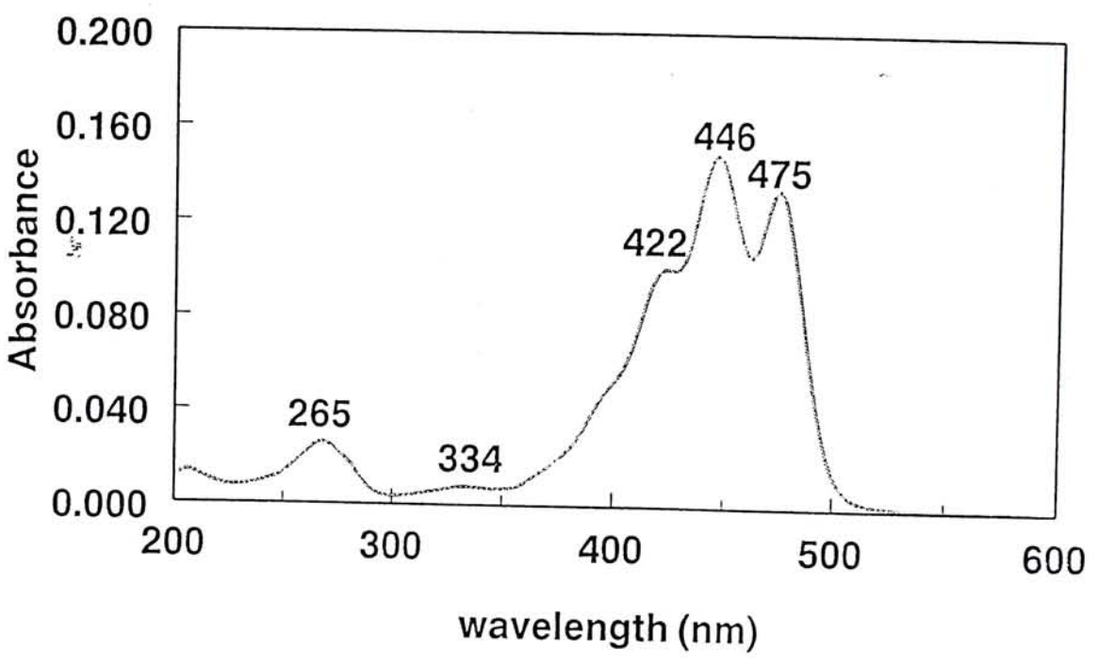

Figure 5b. Absorption spectrum of lutein.

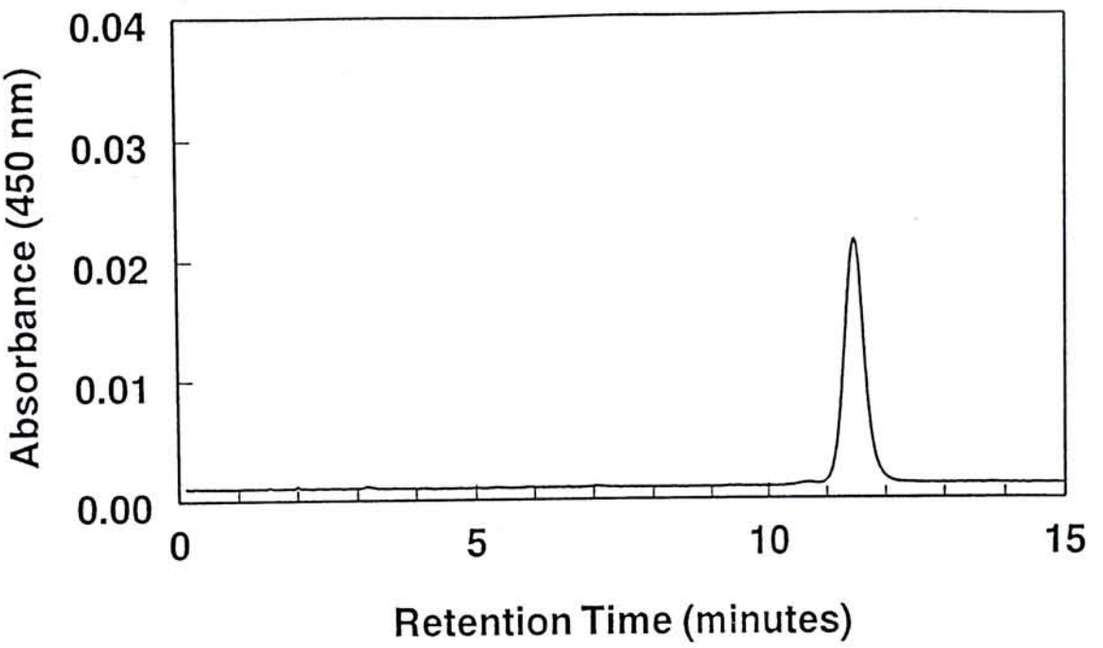

Figure 6a. Chromatogram of zeaxanthin standard at 450 nm.

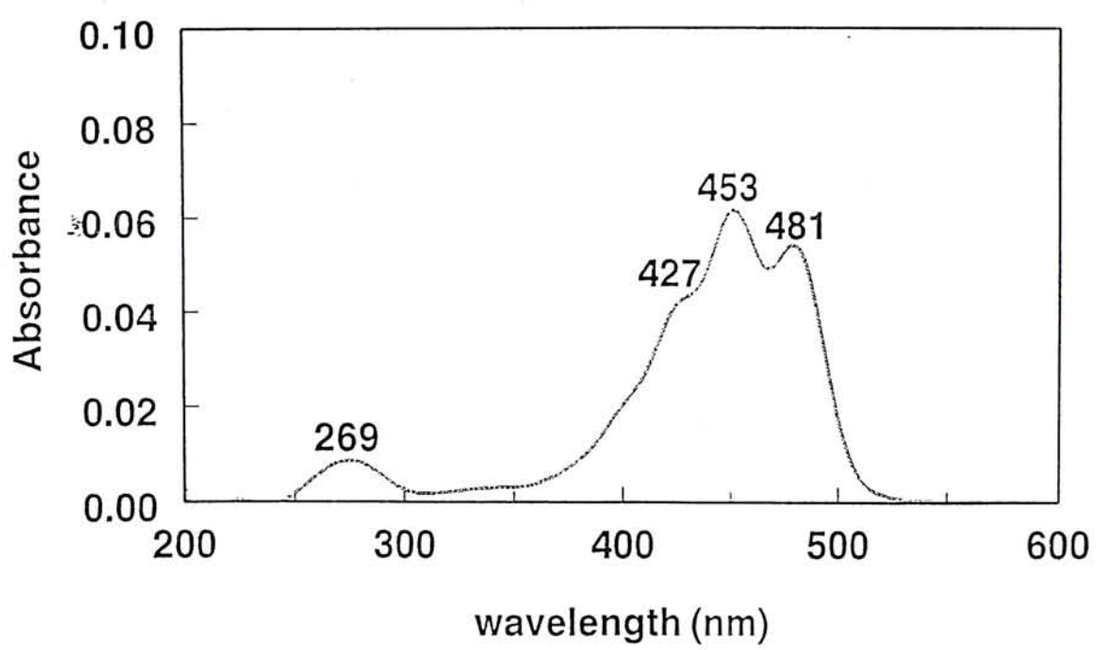

Figure 6b. Absorption spectrum of zeaxanthin .

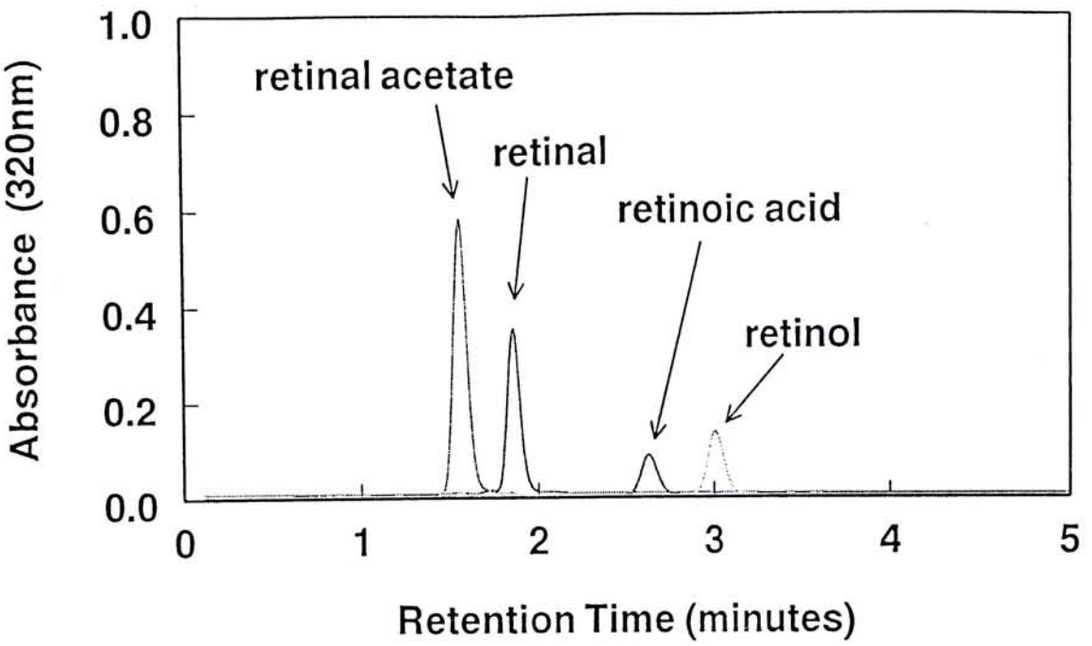

Figure 7a. Chromatographic separation of retinoids.

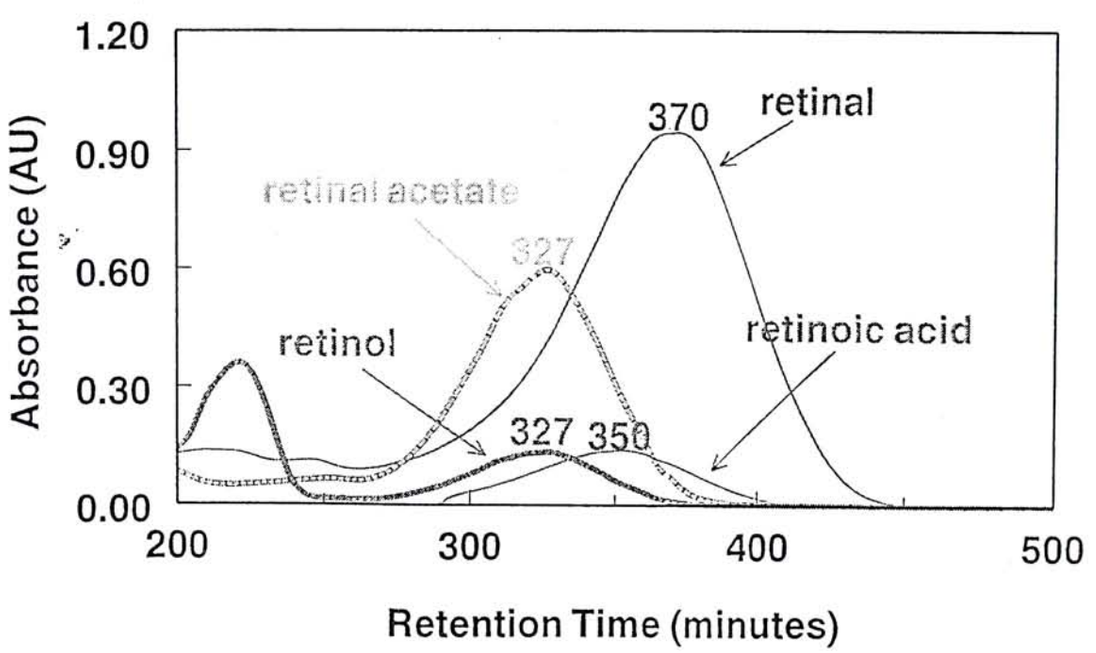

Figure 7b. Absorption spectra taken at the T_r specified in figure 7a.

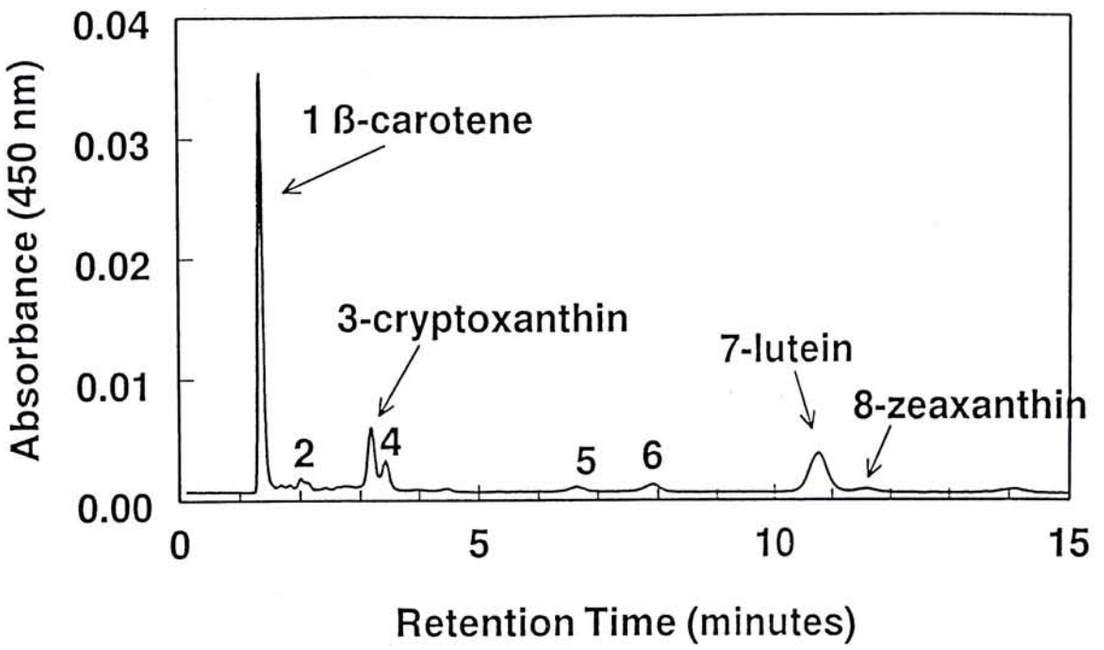

Figure 8a. Chromatogram of the patient's serum at 450 nm.

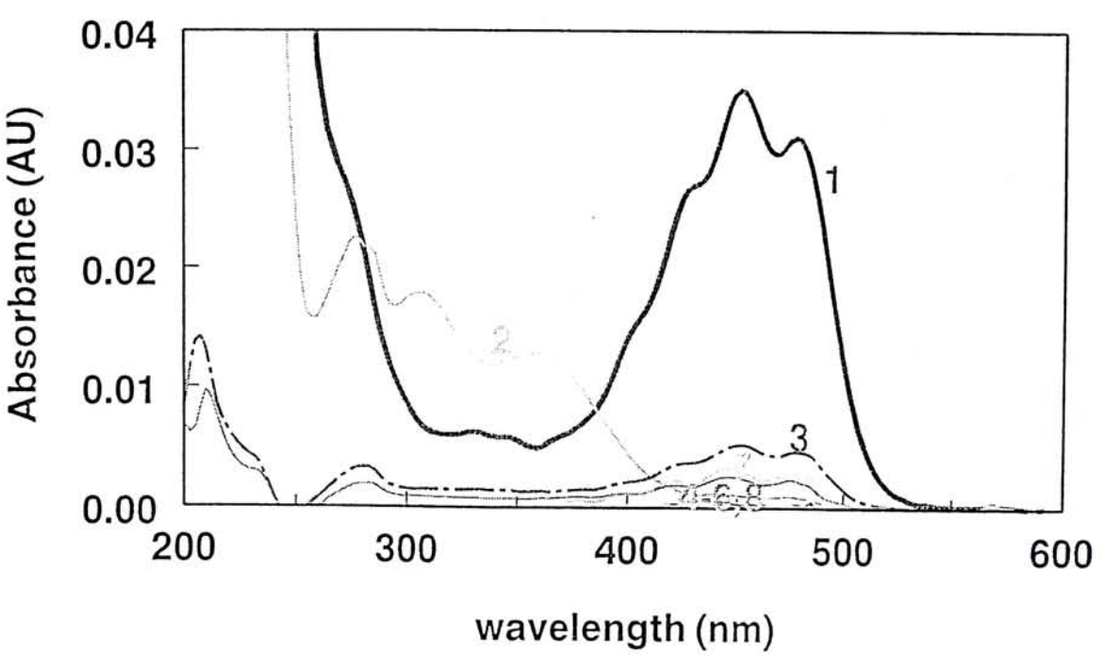

Figure 8b. Absorption spectra of the peaks showed in figure 8a.

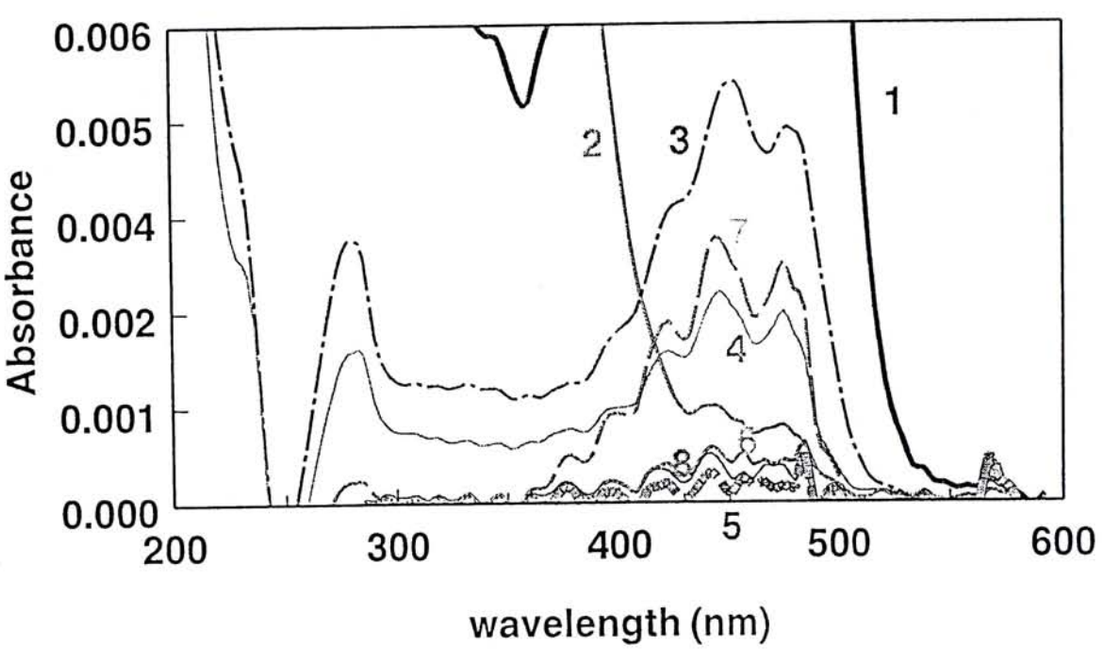

Figure 8c. Highly magnified (x 6.7) absorption spectra of the peaks showed in figure 8b.

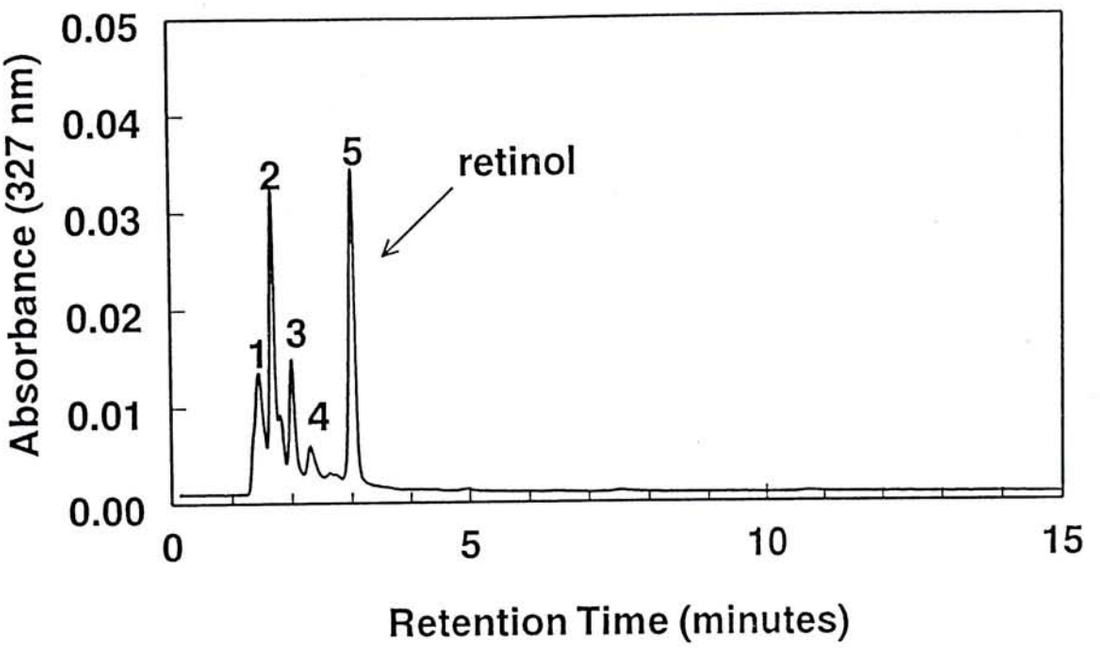

Figure 9a. Chromatogram of human serum at 327 nm.

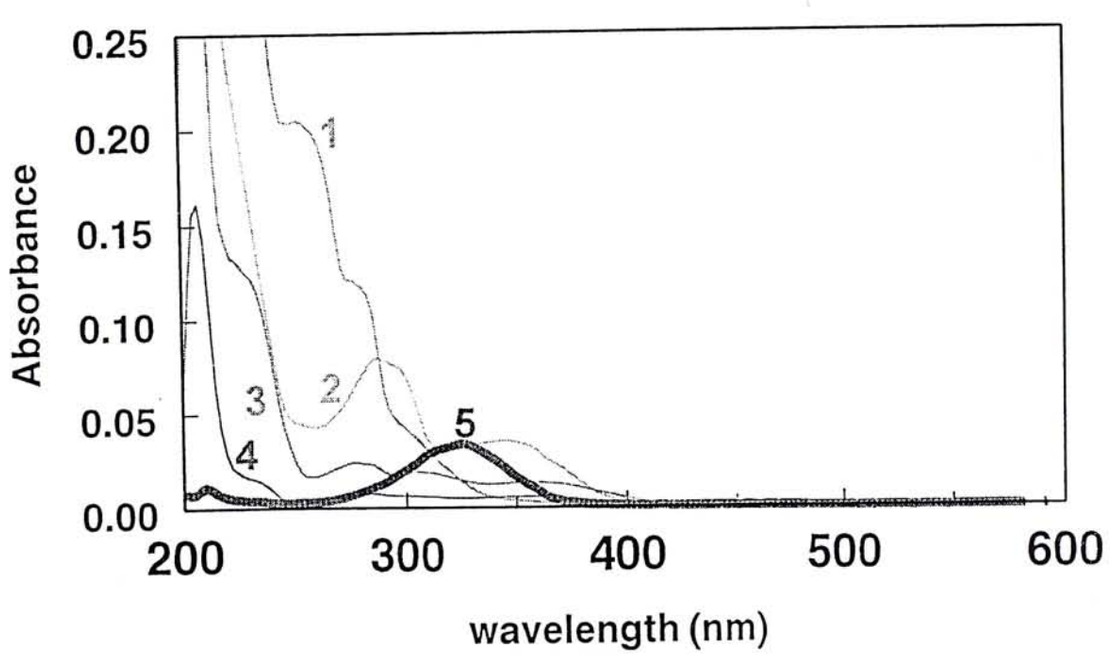

Figure 9b. Absorption spectra of the peaks showed in figure 9a.

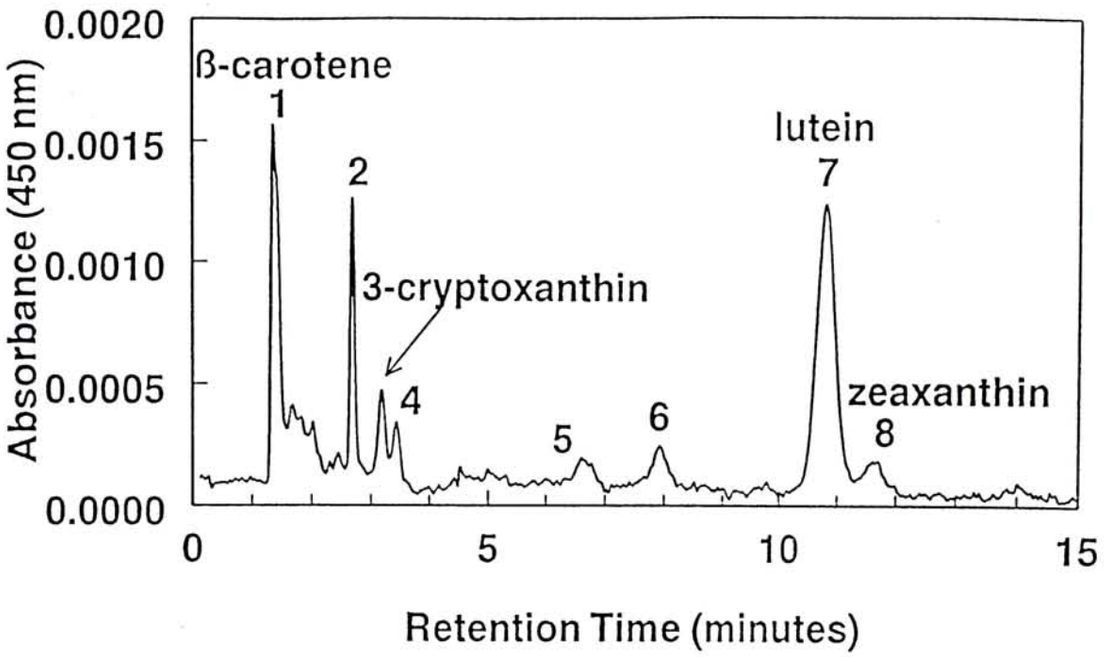

Figure 10a. Chromatogram of subretinal fluid at 450 nm.

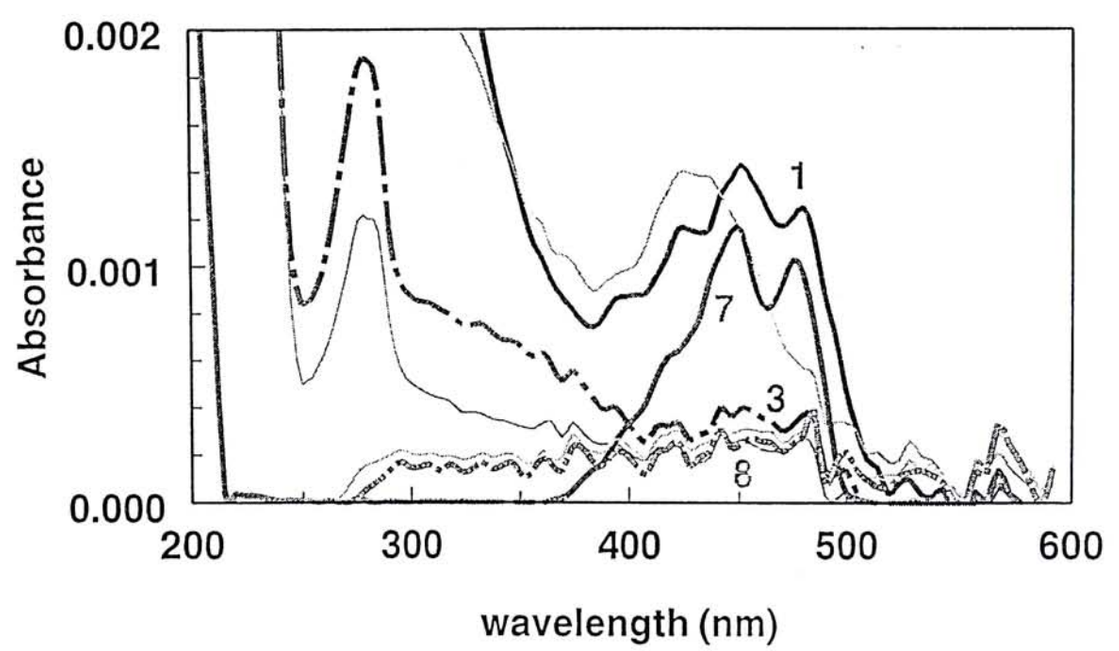

Figure 10b. Absorption spectra of the peaks showed in figure 10a.

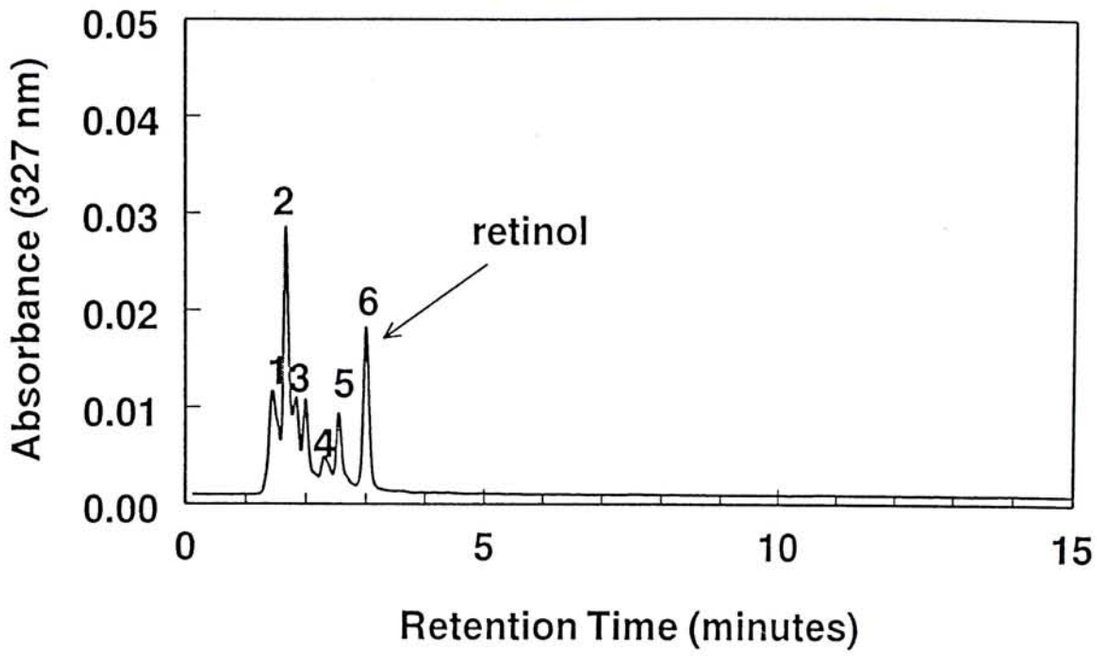

Figure 11a. Chromatogram of subretinal fluid at 327 nm.

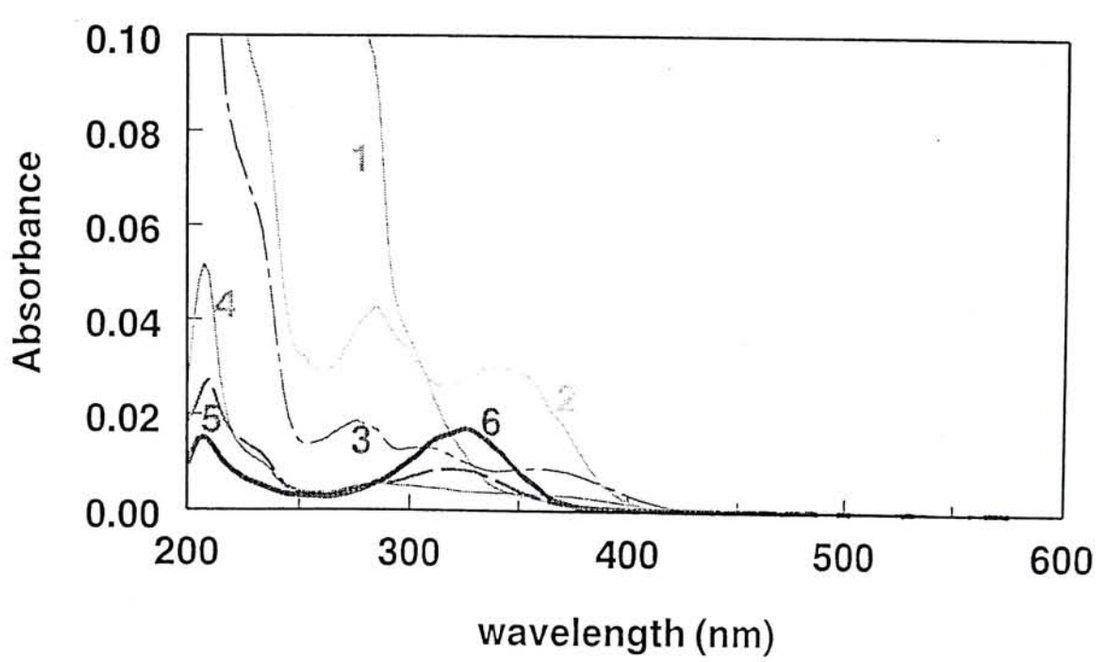

Figure 11b. Absorption spectra of the peaks showed in figure 11b.

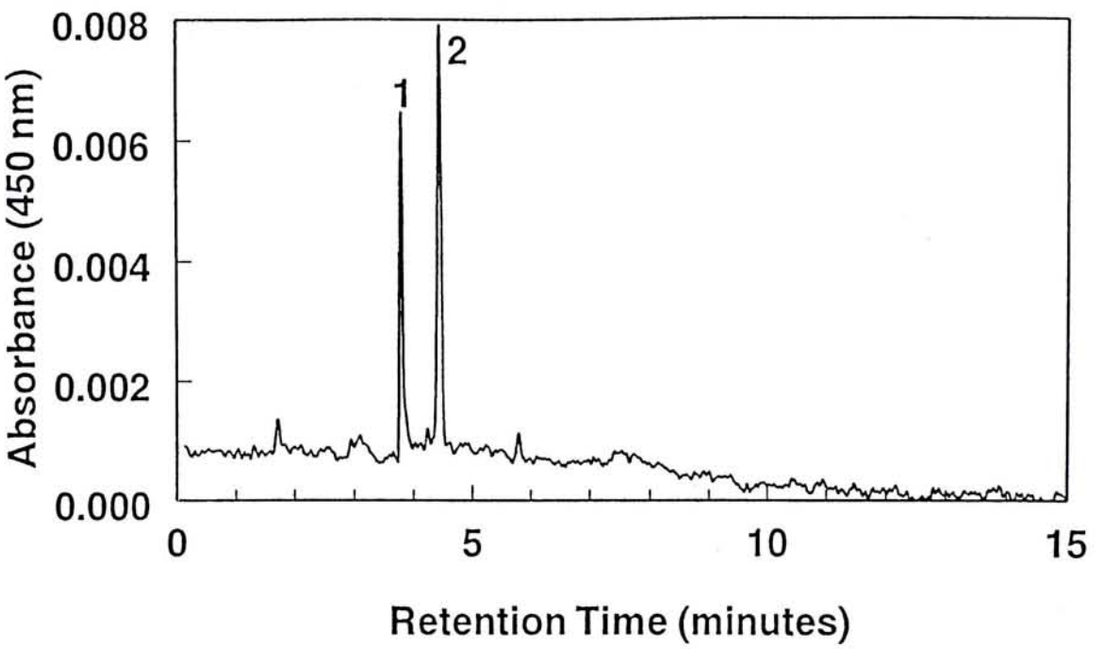

Figure 12a. Chromatogram of liver from rats fed with normal diet at 450 nm.

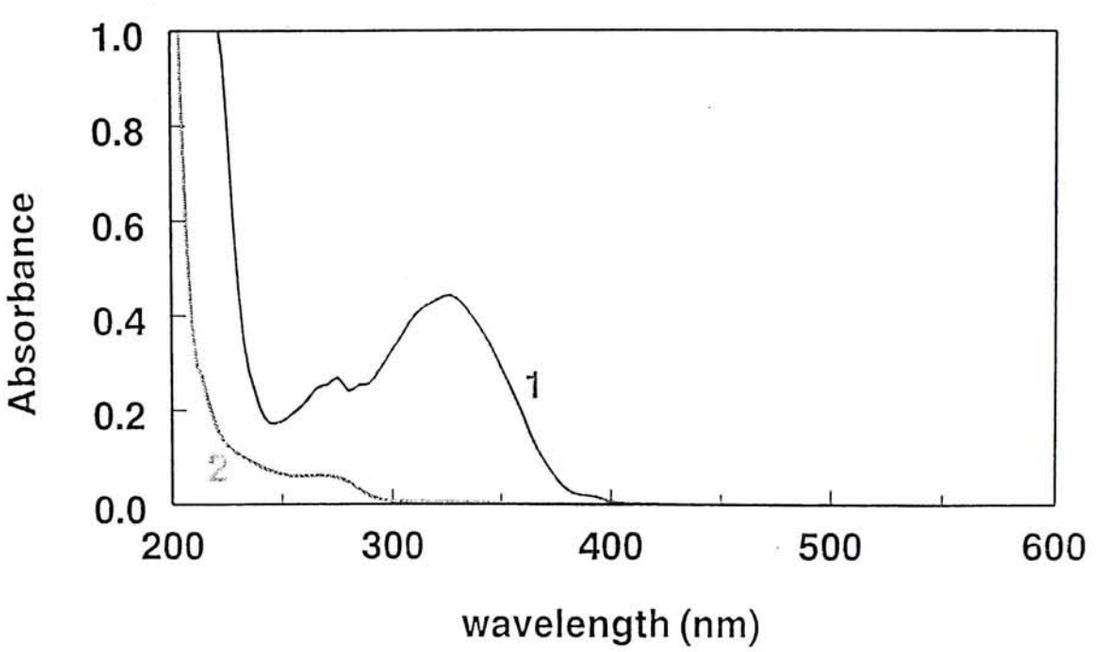

Figure 12b. Absorption spectra of the peaks showed in figure 12a.

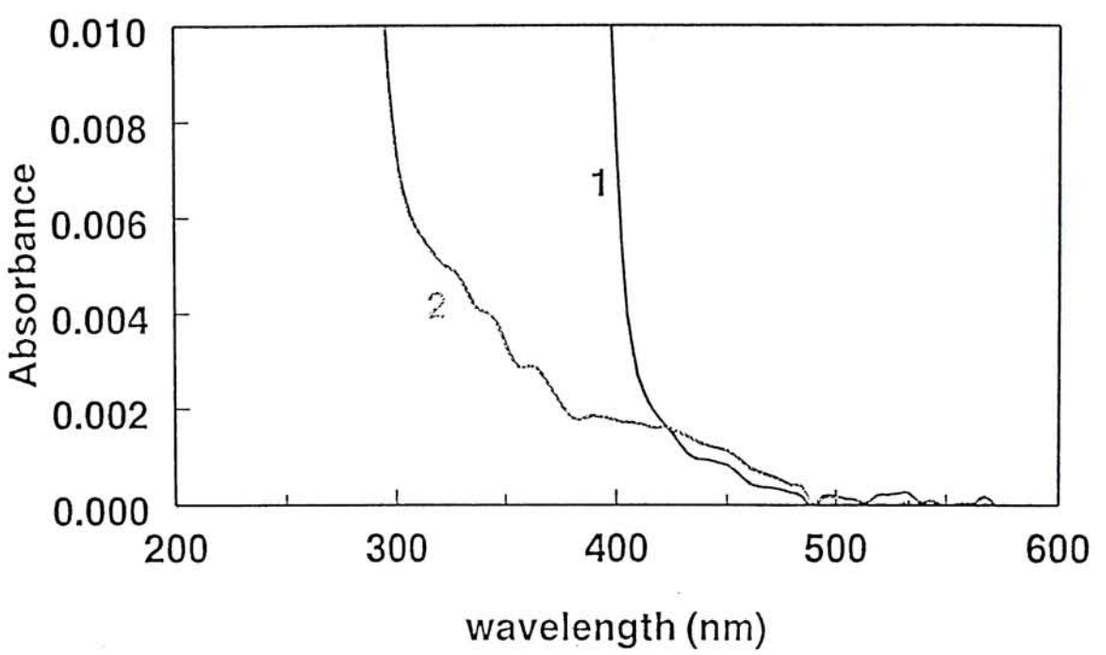

Figure 12c. Highly magnified (x 100) spectra of the peaks in figure 12b.

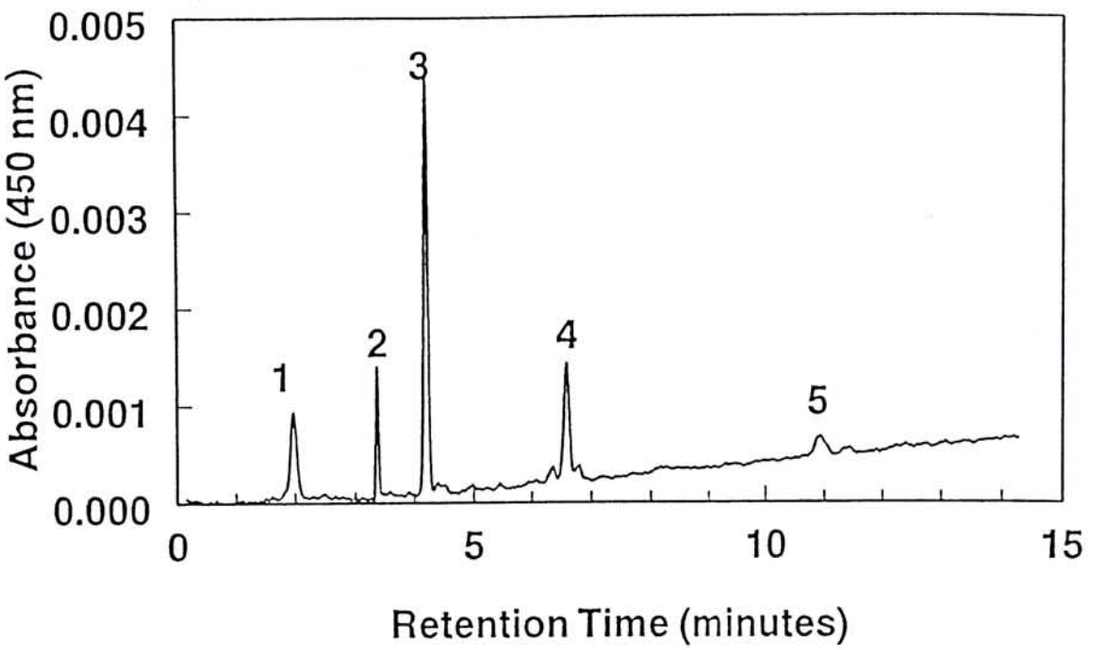

Figure 13a. Chromatogram of liver from rats fed with oil at 450 nm.

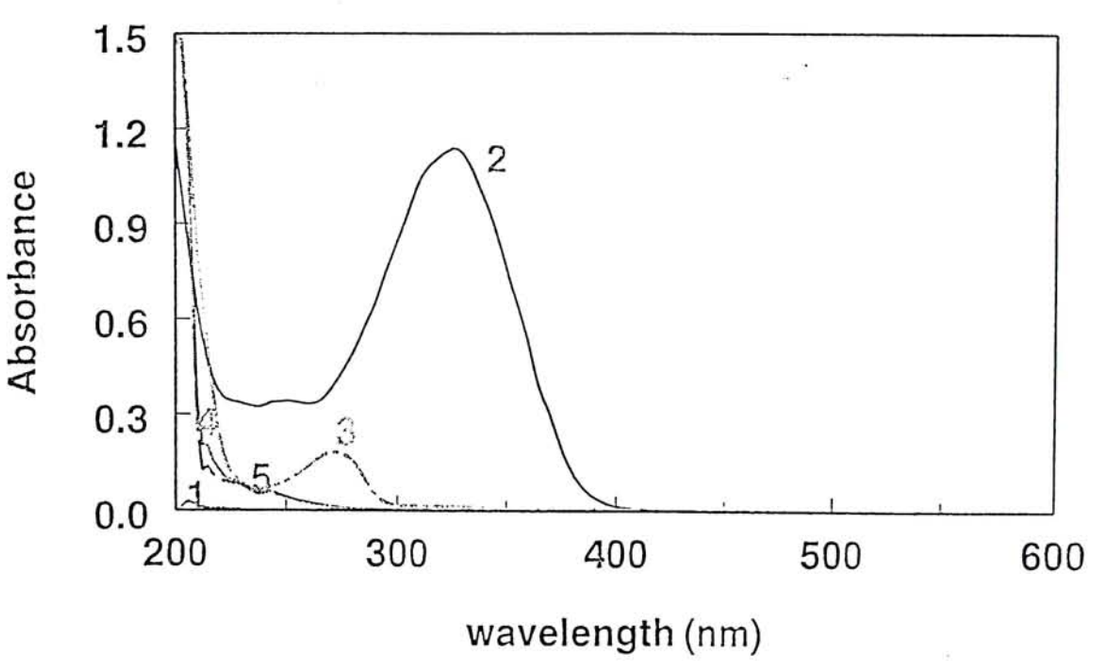

Figure 13b. Absorption spectra of the peaks showed in figure 13a.

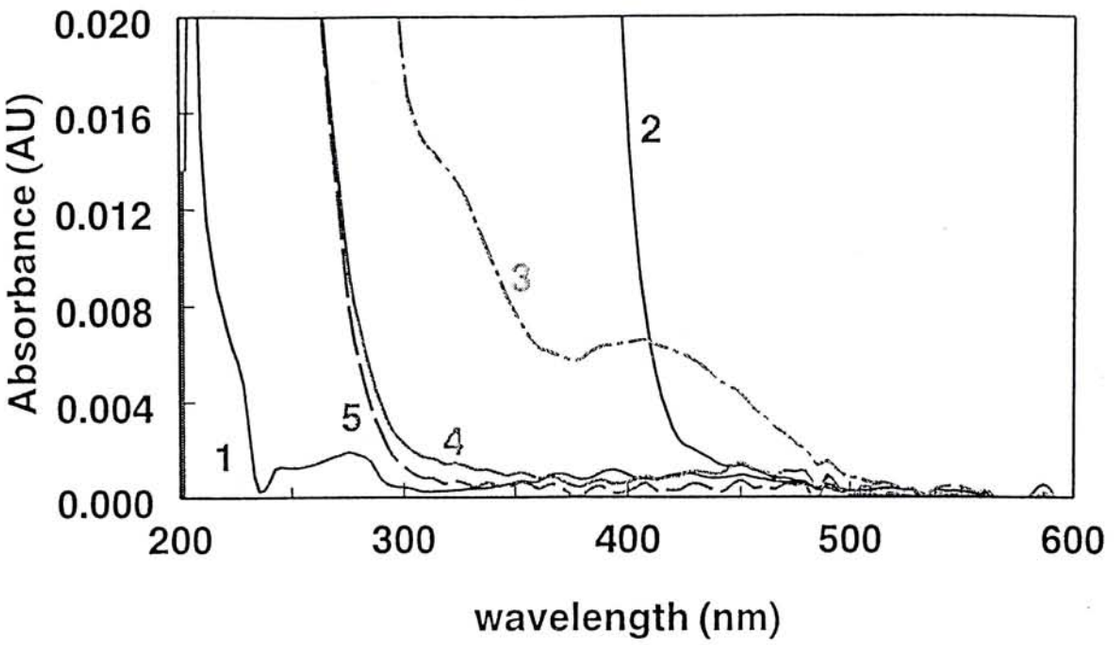

Figure 13c. Higher magnified (x 300) spectra of the peaks showed in figure 13b.

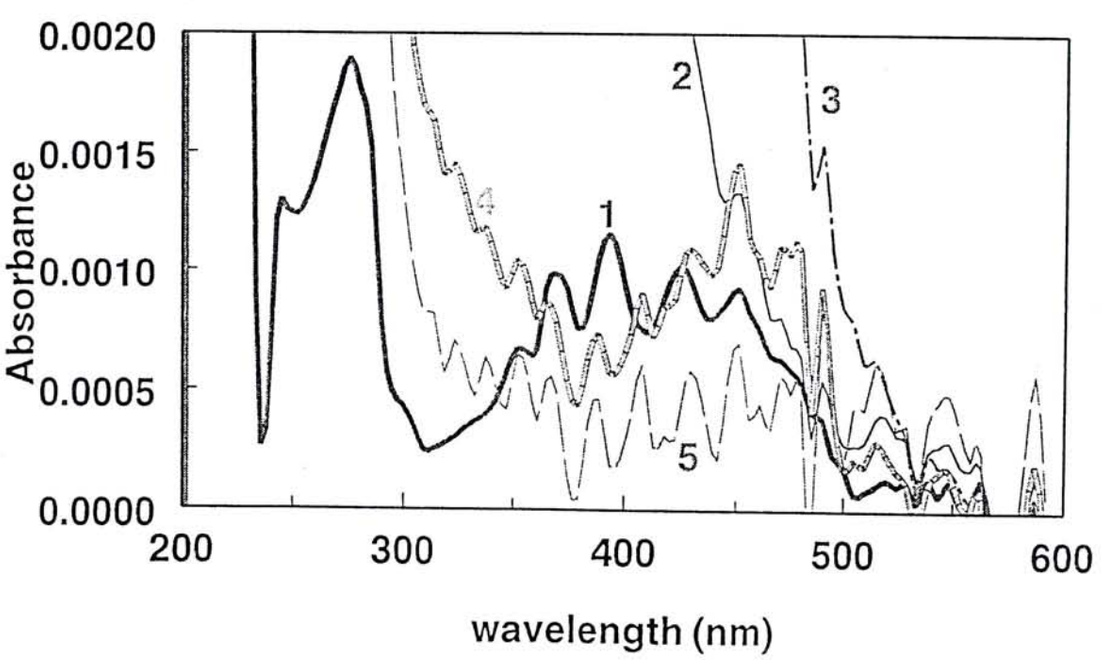

Figure 13d. Further magnified (x 750) spectra of the peaks showed in figure 13b.

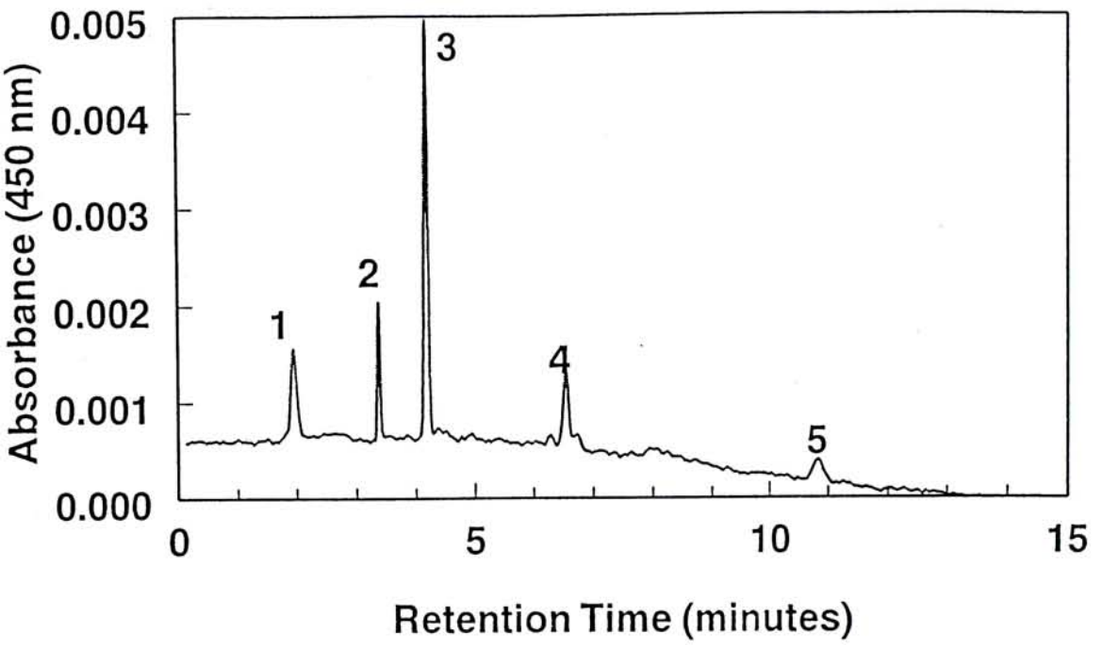

Figure 14a. Chromatogram of liver from rat fed with lutein (1mg/kg) at 450 nm.

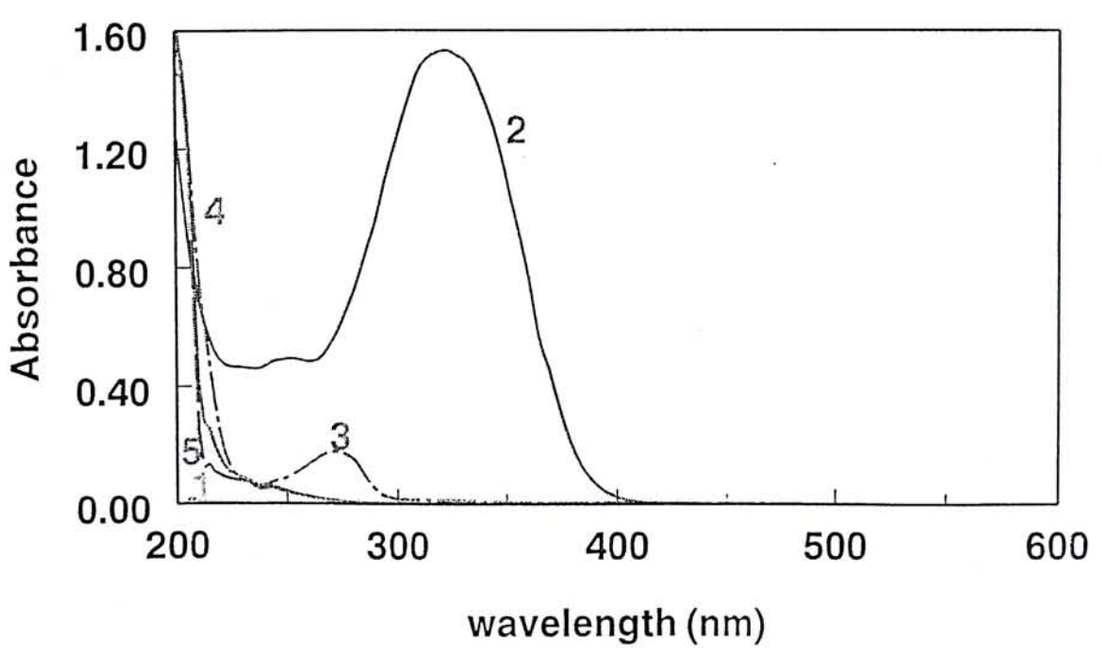

Figure 14b. Absorption spectra of the peaks showed in figure 14a.

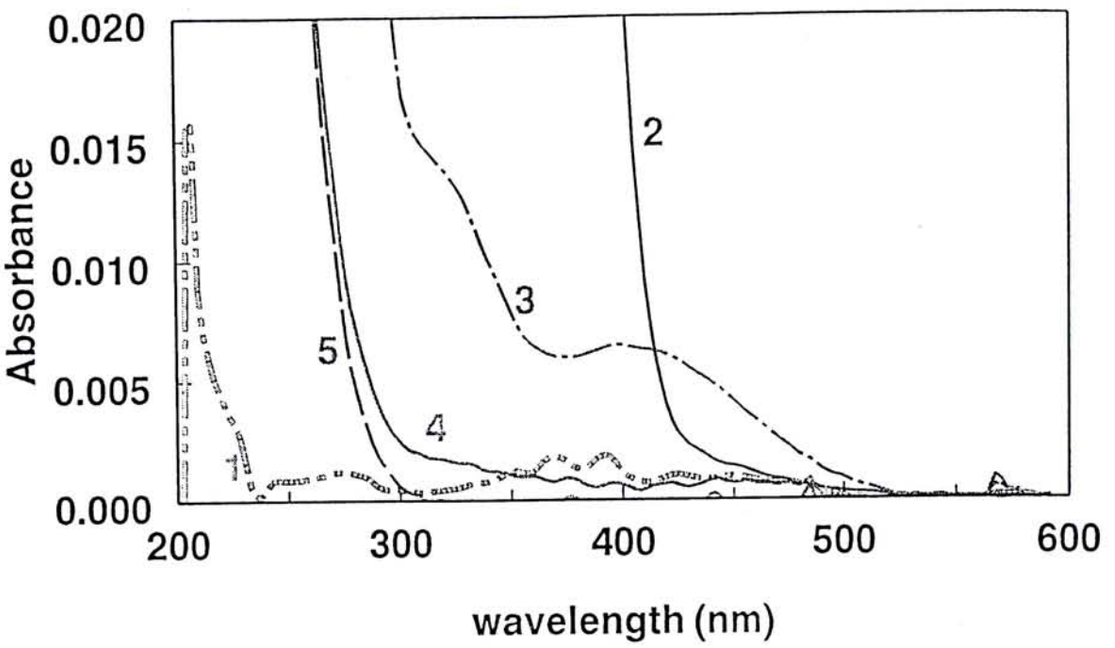

Figure 14c. Highly magnified (x 80) spectra of the peaks showed in figure 14b.

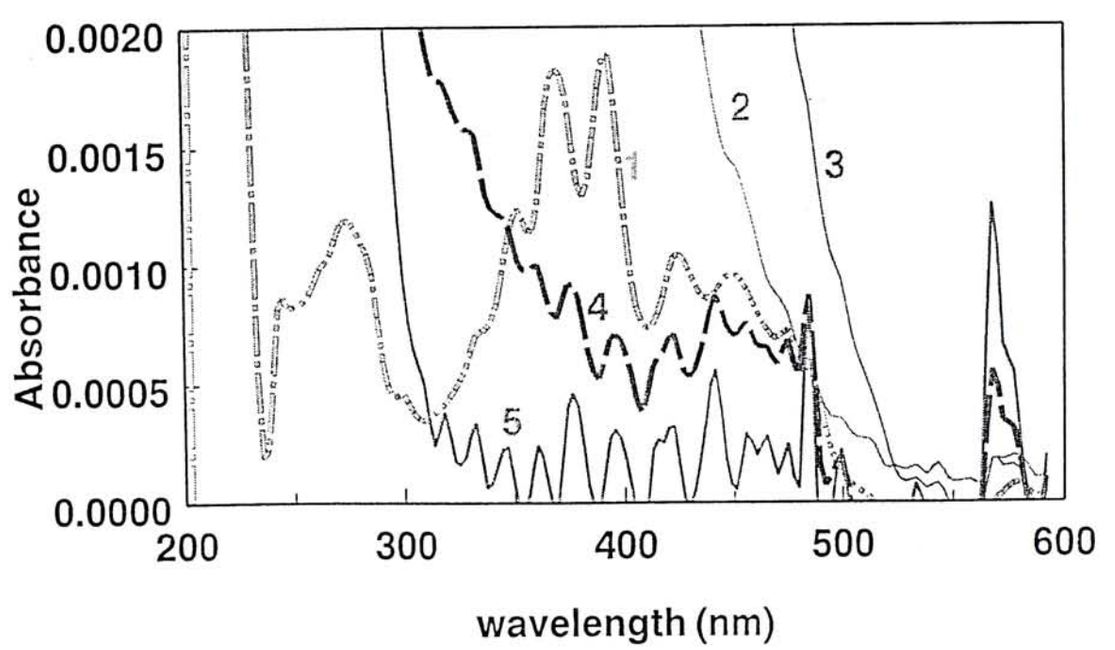

Figure 14d. Further magnified (x 800) spectra of the peaks showed in figure 14b.

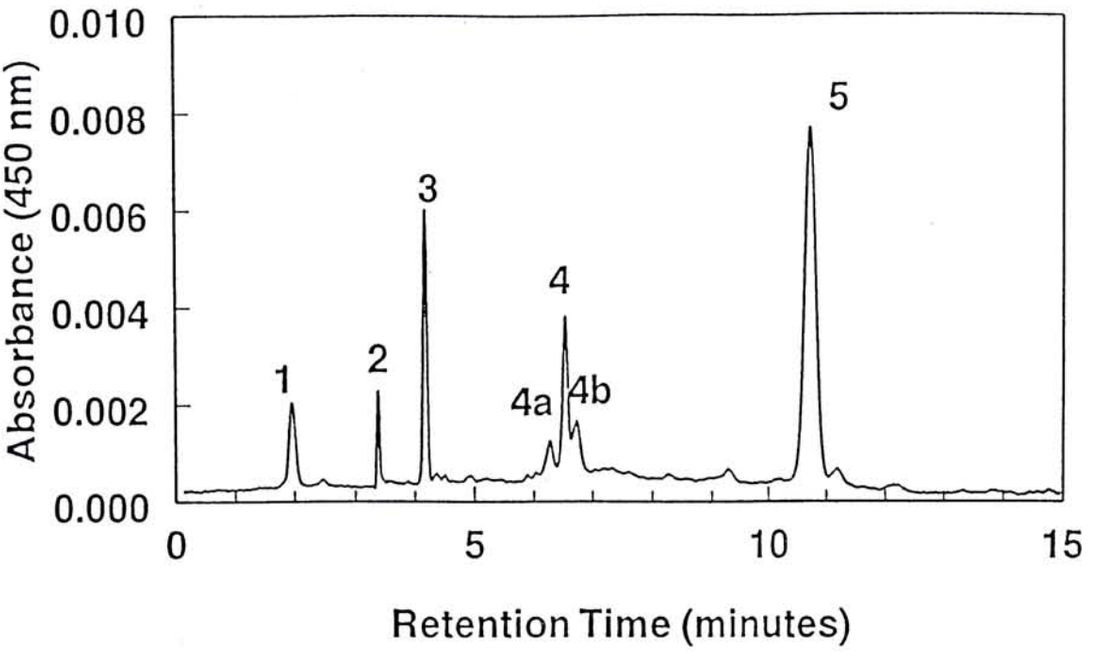

Figure 15a. Chromatogram of liver from rat fed with lutein (30mg/kg) at 450 nm.

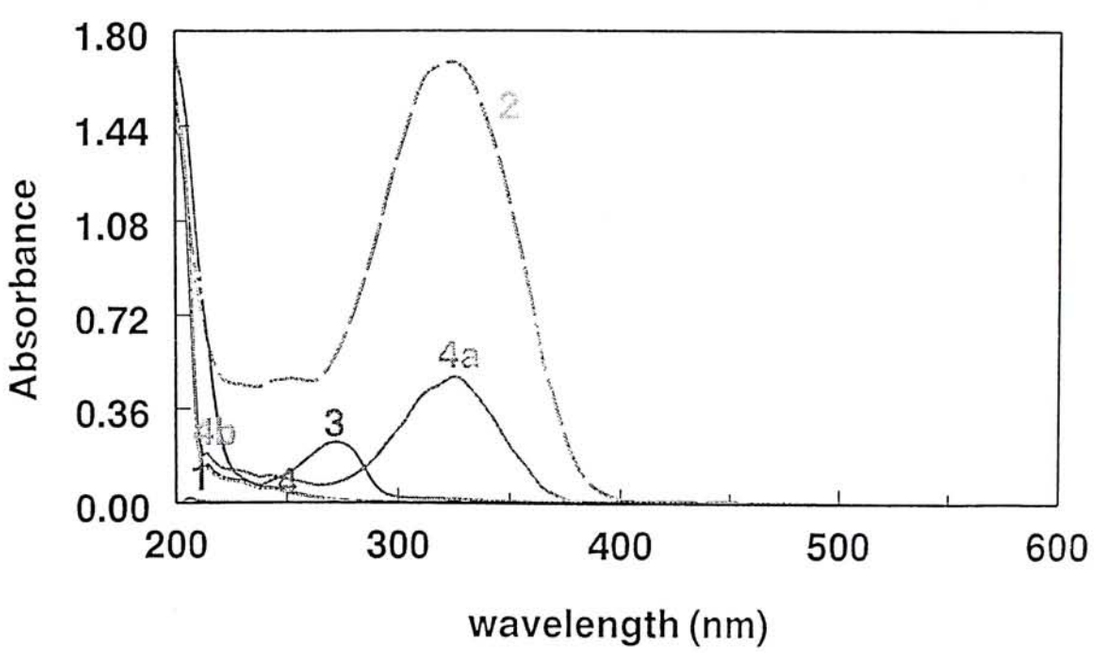

Figure 15b. Absorption spectra of the peaks showed in figure 15a.

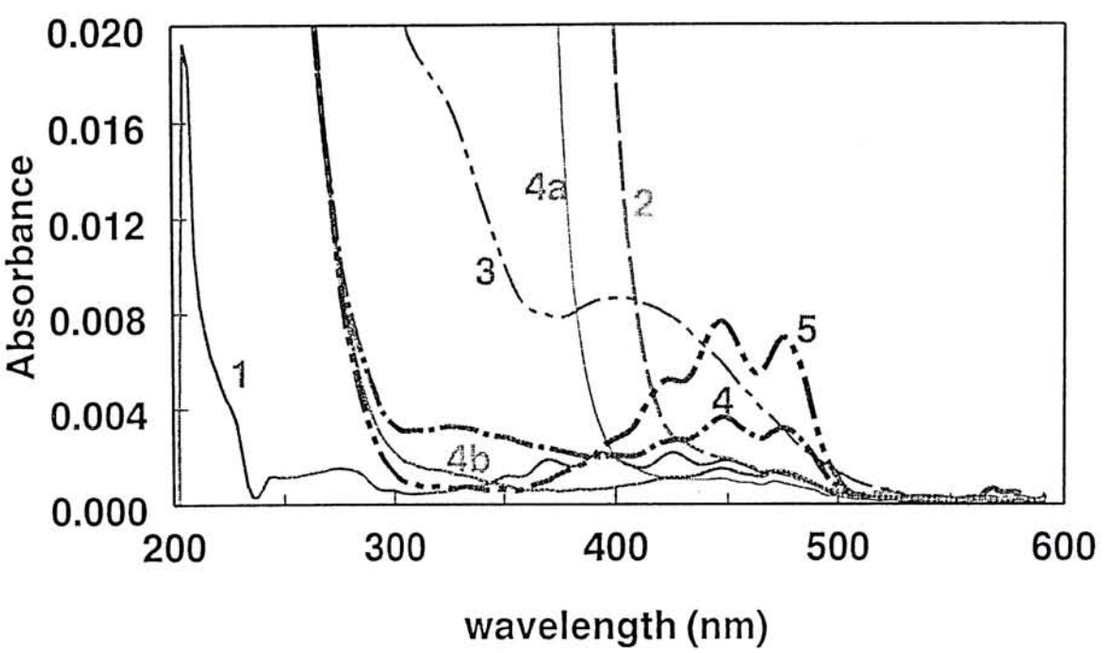

Figure 15c. Highly magnified (x 180) absorption spectra of the peaks showed in figure 15b.

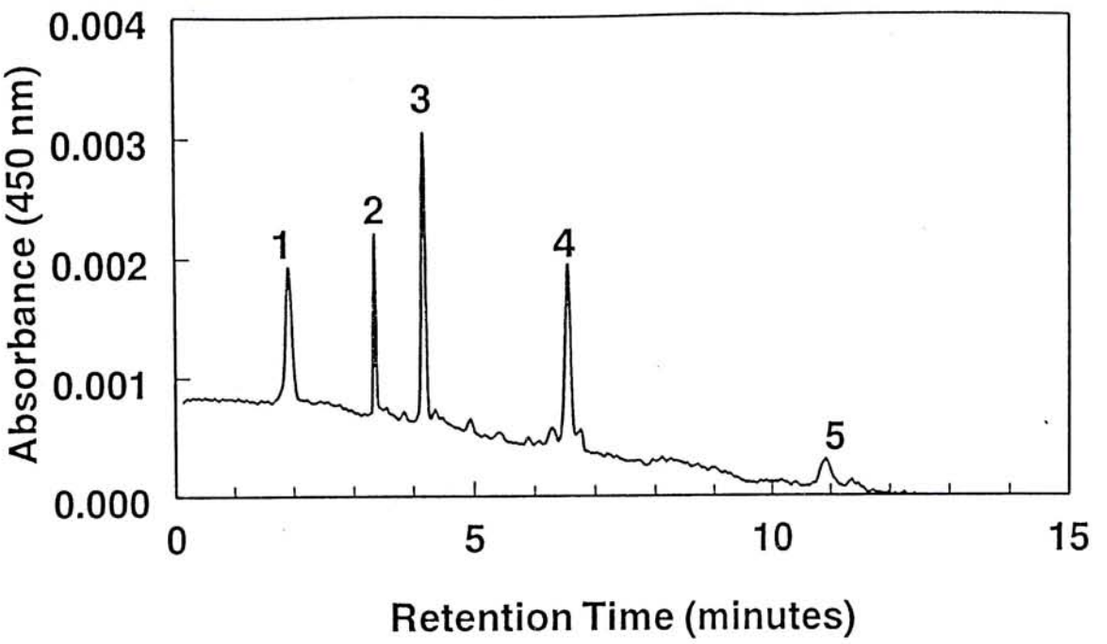

Figure 16a. Chromatogram of liver from rat fed with astaxanthin (1mg/kg) at 450 nm .

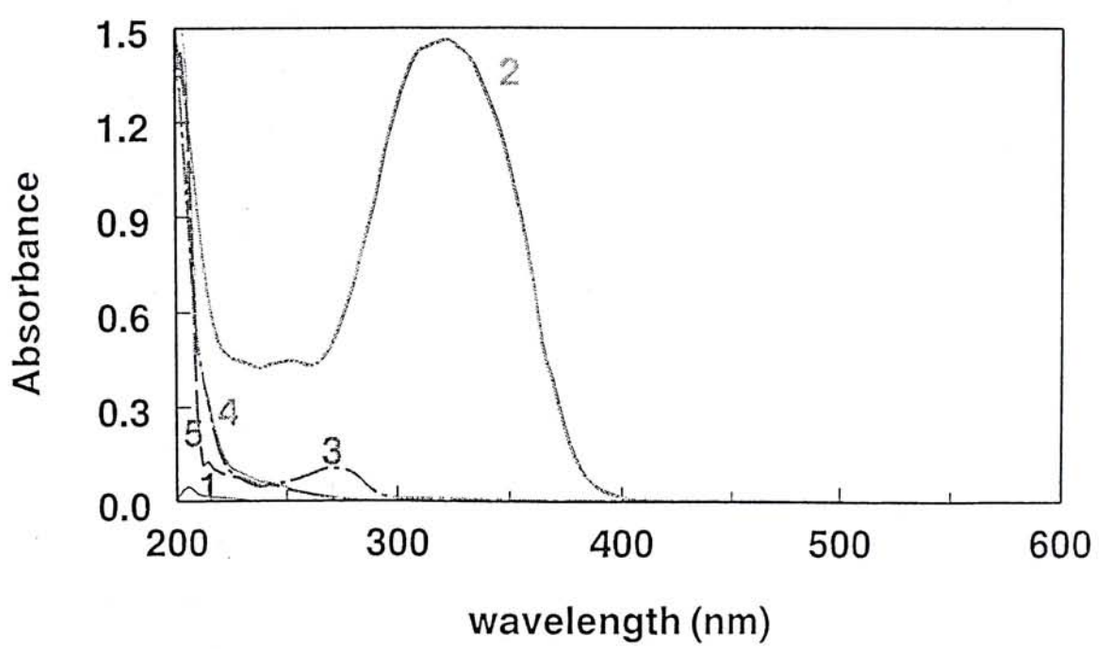

Figure 16b. Absorption spectra of the peaks showed in figure 16a.

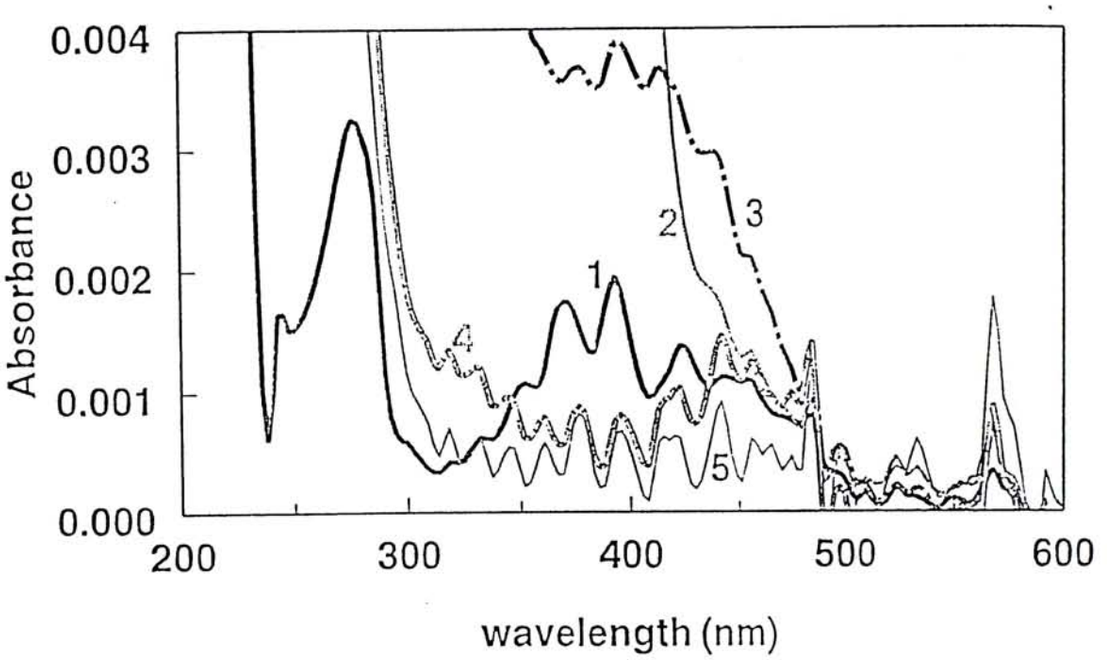

Figure 16c. Highly magnified (x 375) absorption spectra of the peaks showed in figure 16b.

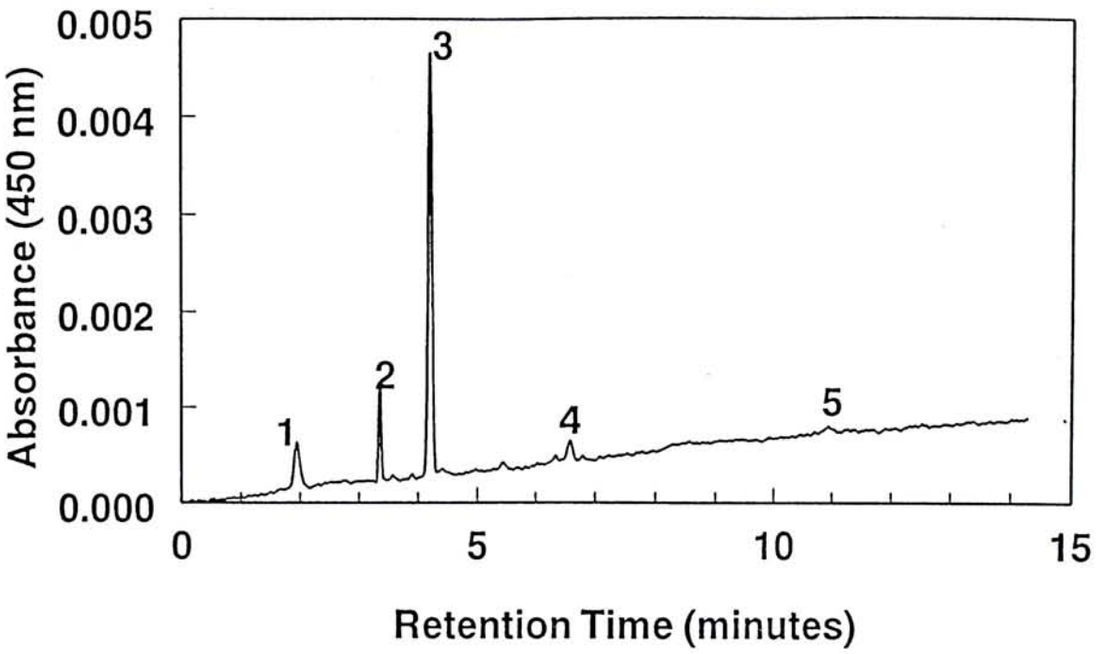

Figure 17a. Chromatogram of liver from rat fed with astaxanthin (10mg/kg) at 450 nm.

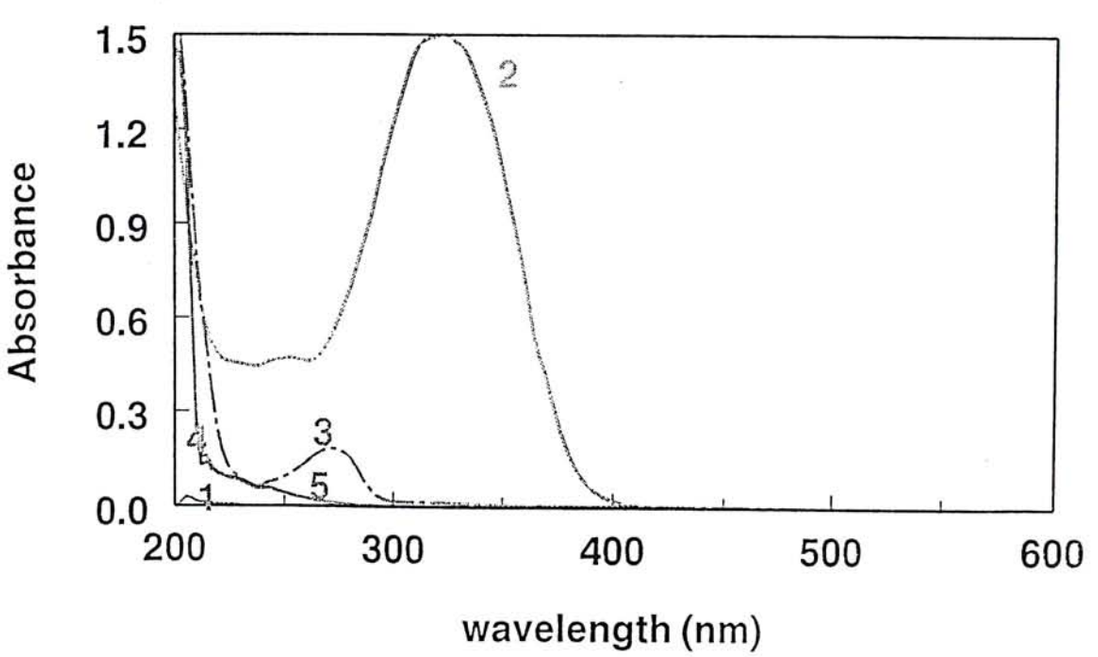

Figure 17b. Absorption spectra of the peaks showed in figure 17a.

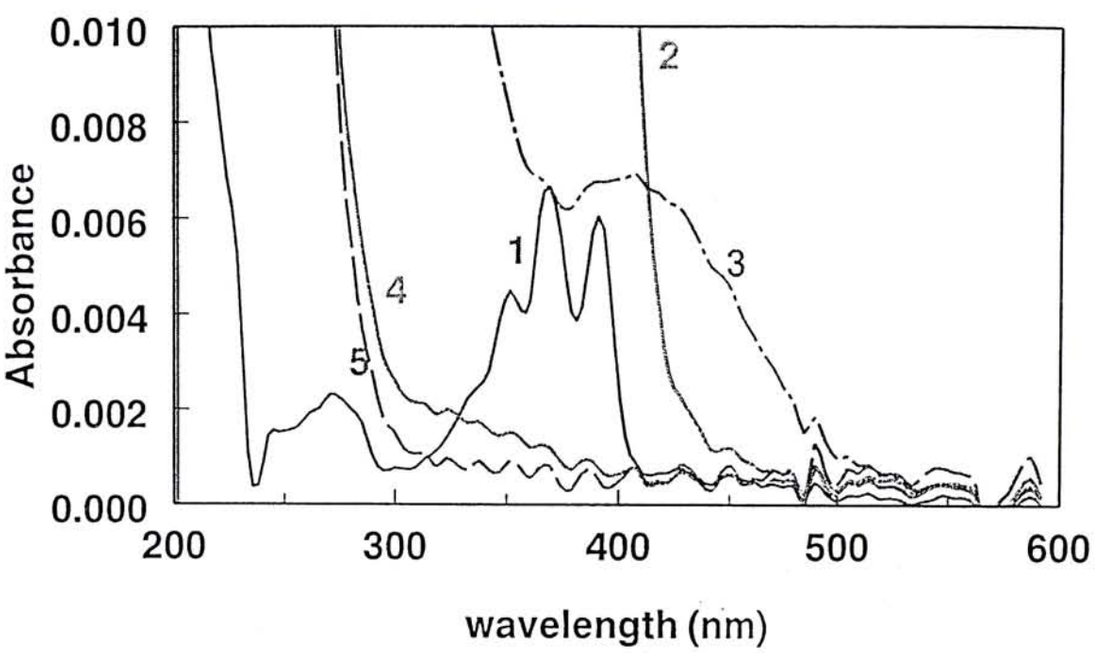

Figure 17c. Highly magnified (x 150) absorption spectra of the peaks showed in figure 17b.

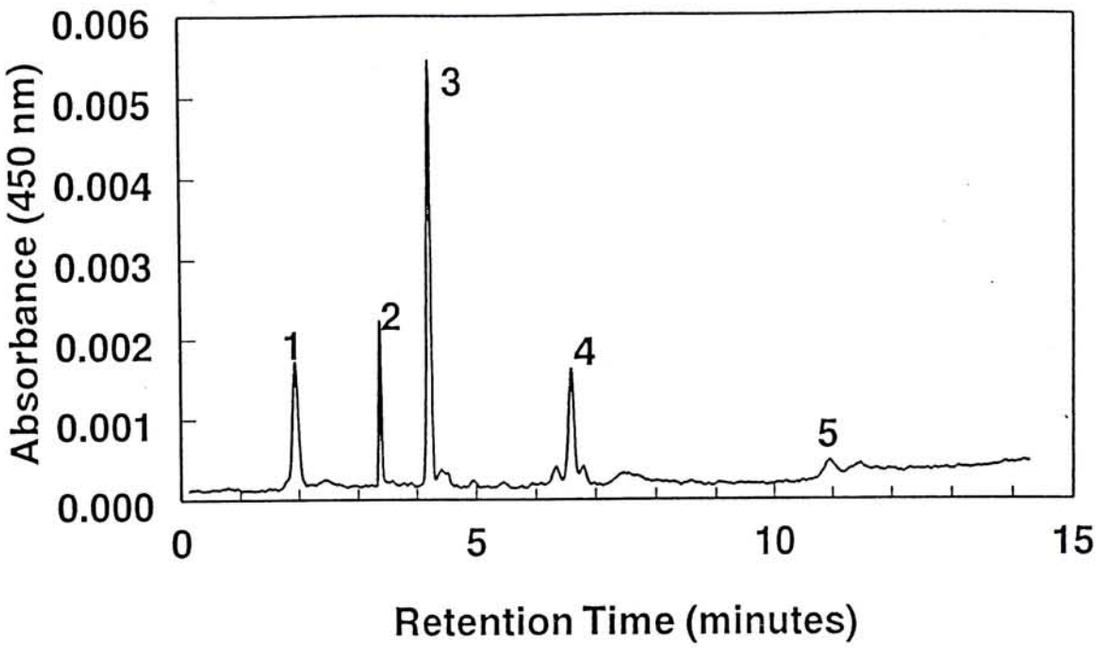

Figure 18a. Chromatogram of liver from rat fed with astaxanthin (30mg/kg) at 450 nm .

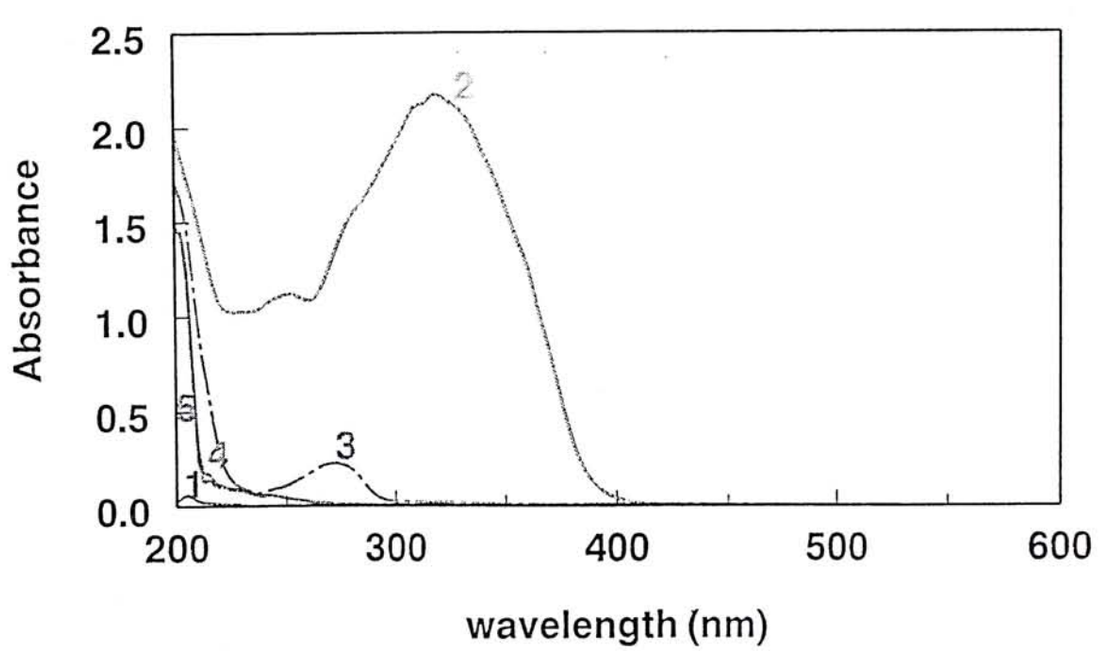

Figure 18b. Absorption spectra of the peaks showed in figure 18a.

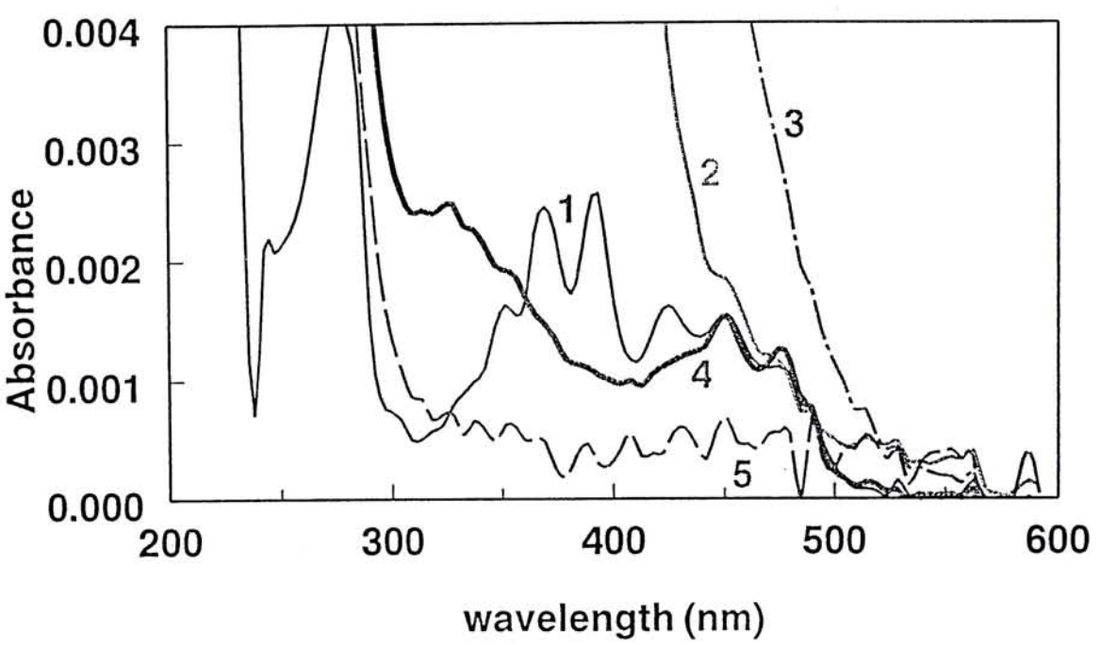

Figure 18c. Higher magnified (x 625) absorption spectra of the peaks showed in figure 18b.

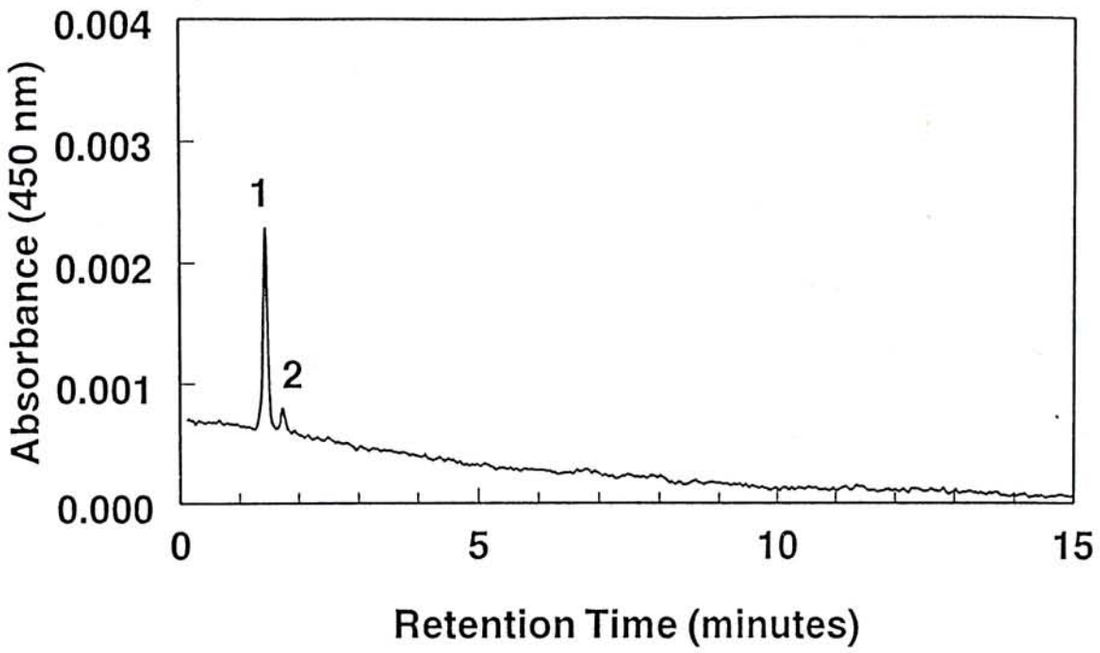

Figure 19a. Chromatogram of serum from rats fed with normal diet at 450 nm .

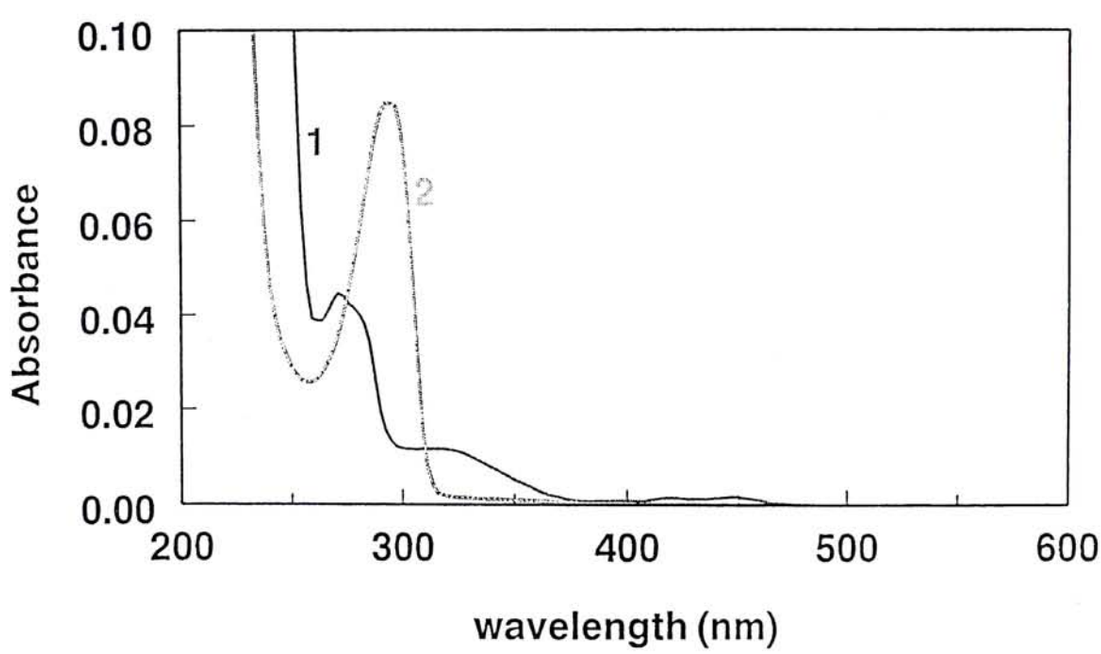

Figure 19b. Absorption spectra of the peaks showed in figure 19a.

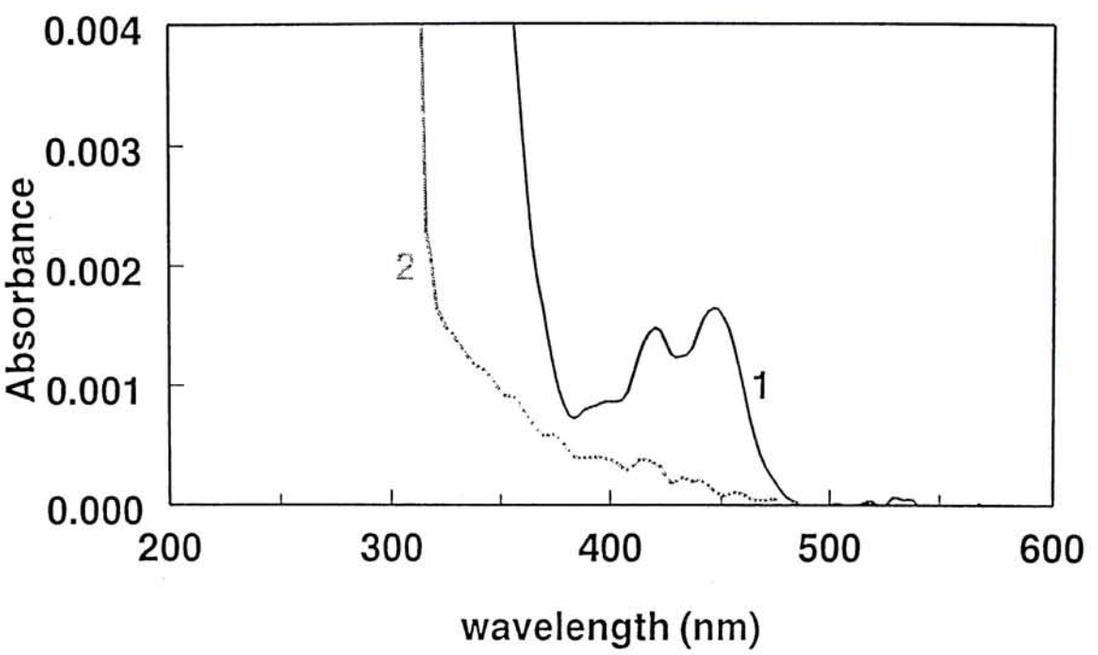

Figure 19c. Highly magnified (x 20) absorption spectra of the peaks showed in figure 19b.

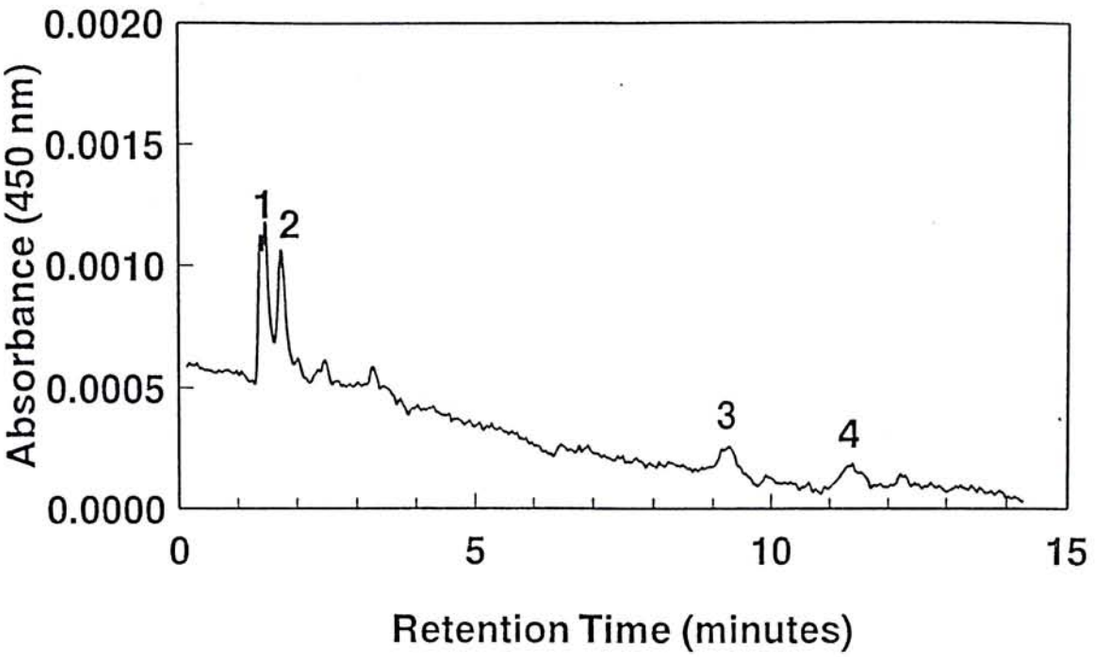

Figure 20a. Chromatogram of serum from rats fed with oil at 450 nm.

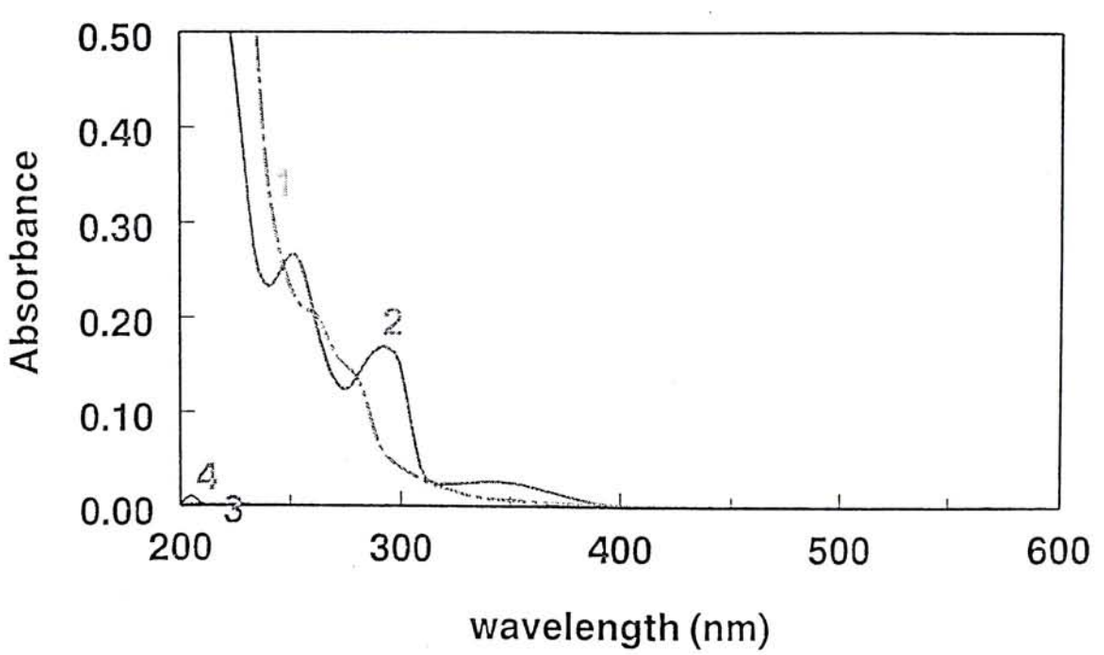

Figure 20b. Absorption spectra of the peaks showed in figure 20a.

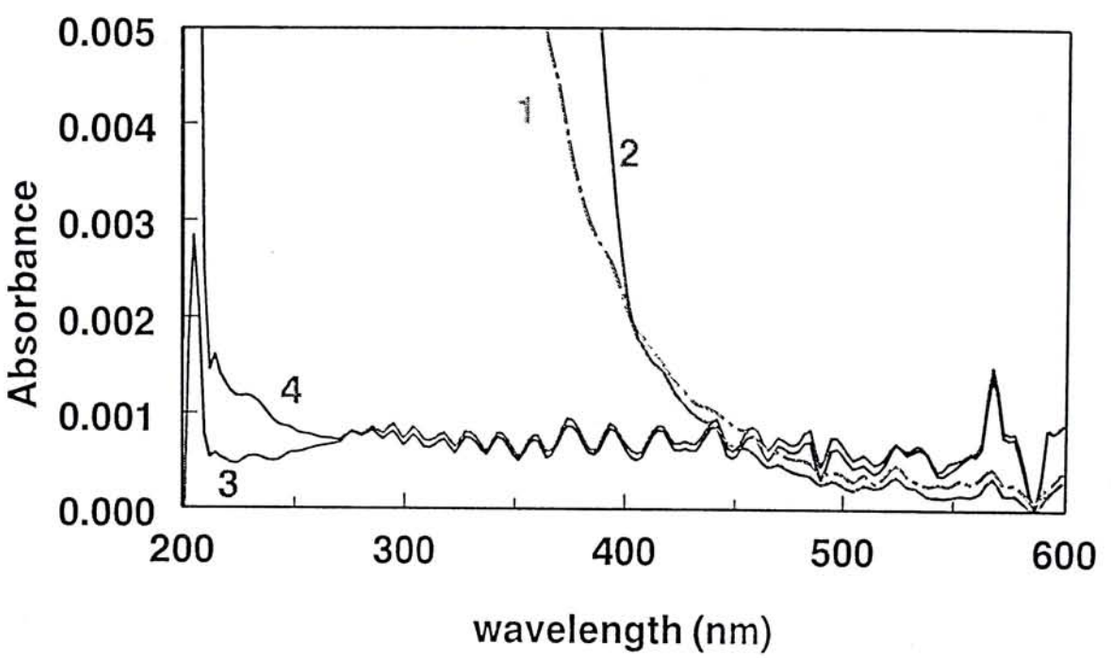

Figure 20c. Highly magnified (x 100) absorption spectra of the peaks showed in figure 20b.

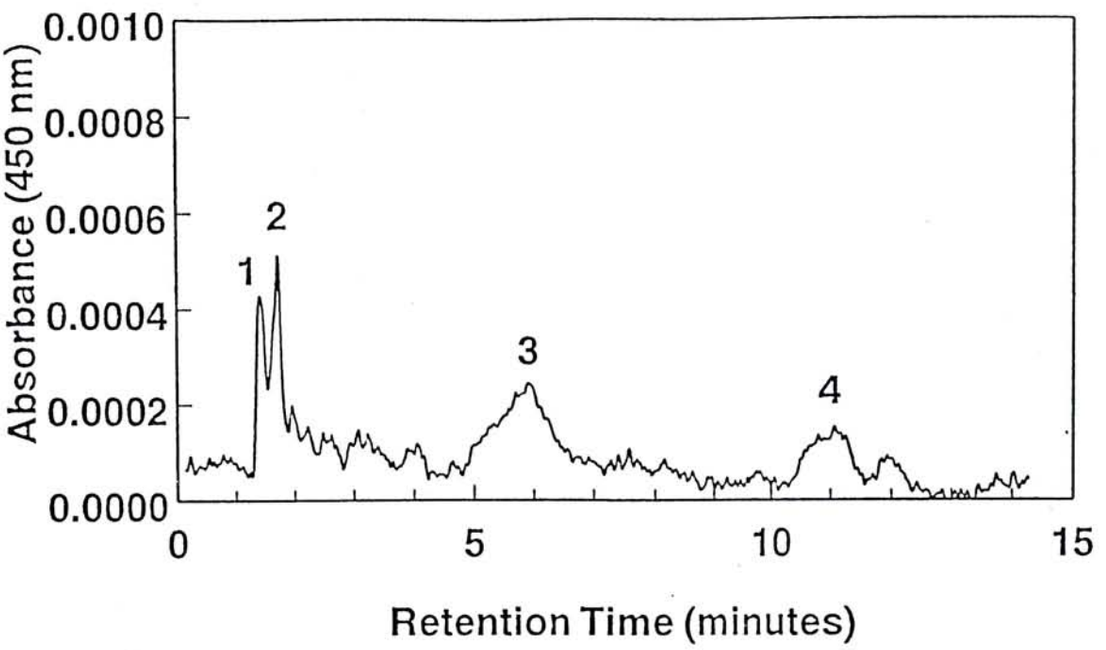

Figure 21a. Chromatogram of serum from rats fed with lutein (1mg/kg) at 450 nm.

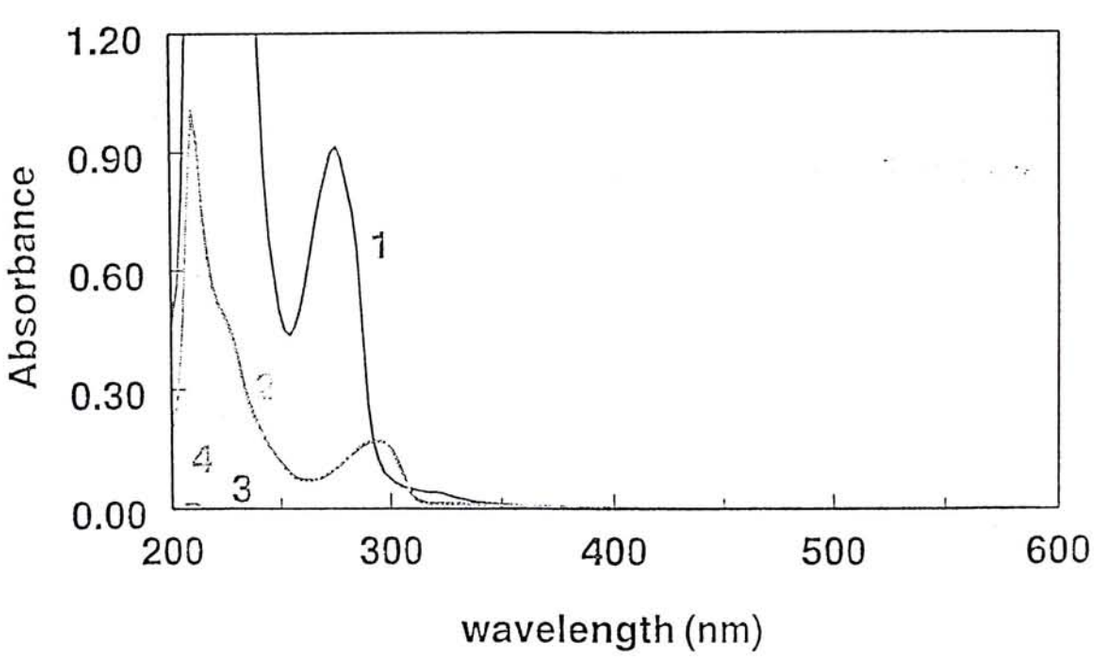

Figure 21b. Absorption spectra of the peaks showed in figure 21a.

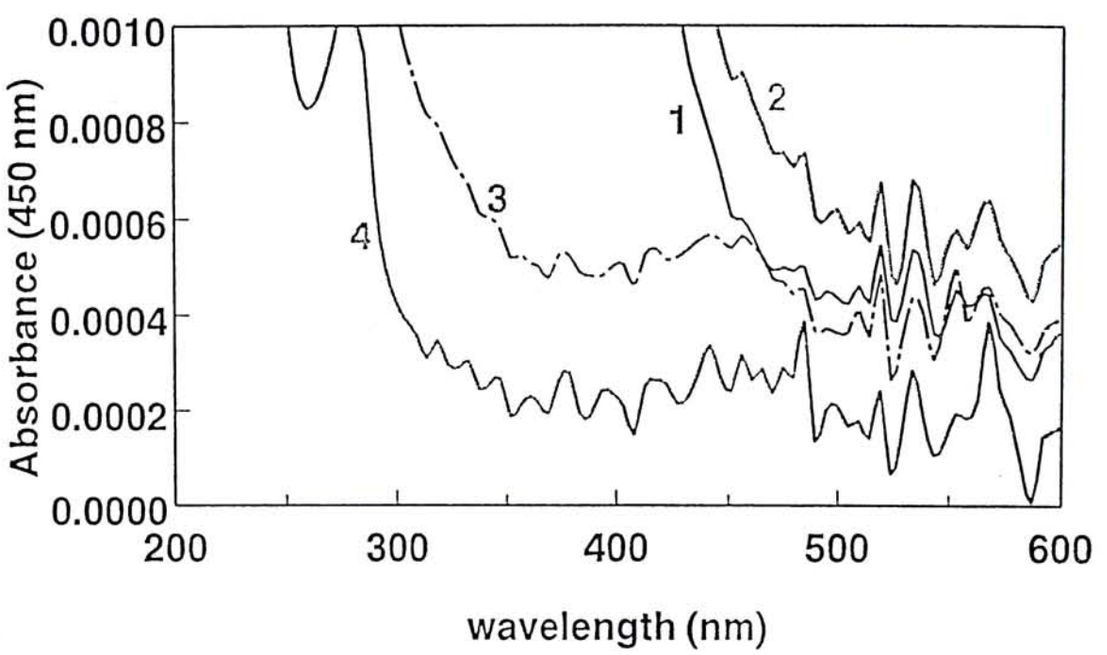

Figure 21c. Highly magnified (x 800) absorption spectra of the peaks showed in figure 21b.

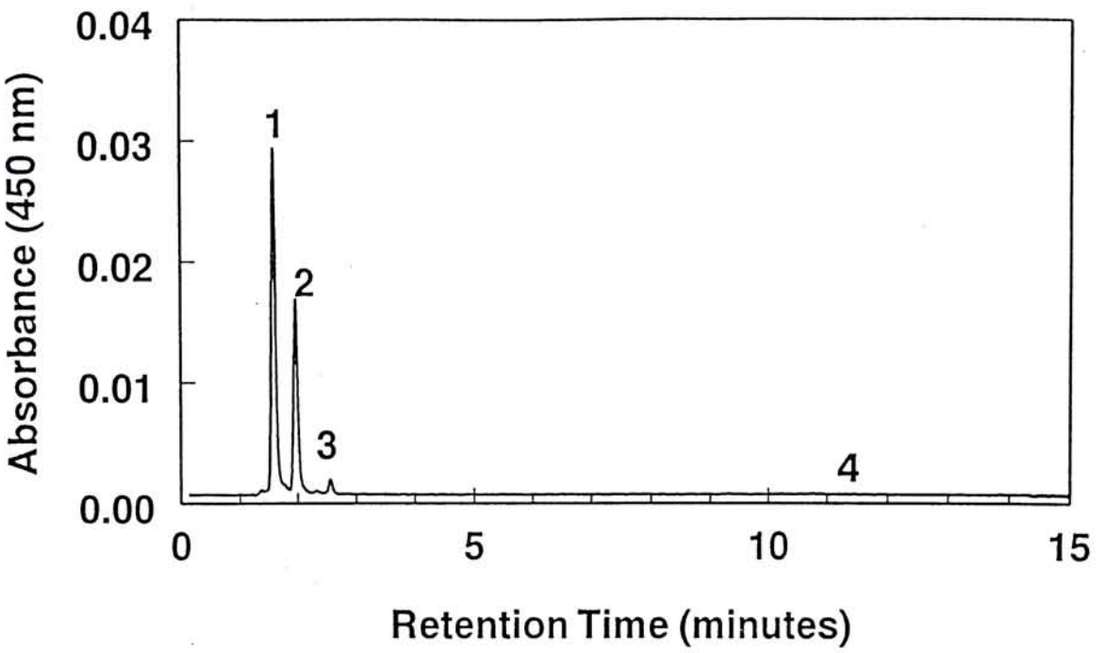

Figure 22a. Chromatogram of serum from rat fed with lutein (10mg/kg) at 450 nm.

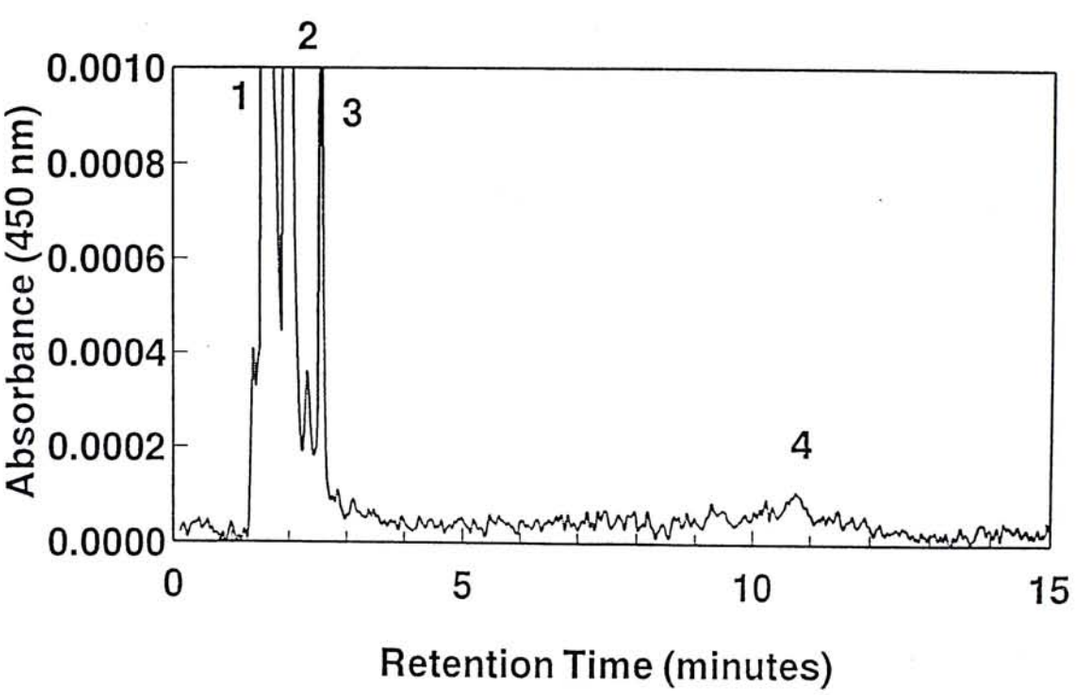

Figure 22b. Highly magnified (x 40) chromatogram of the absorption peaks showed in figure 22a.

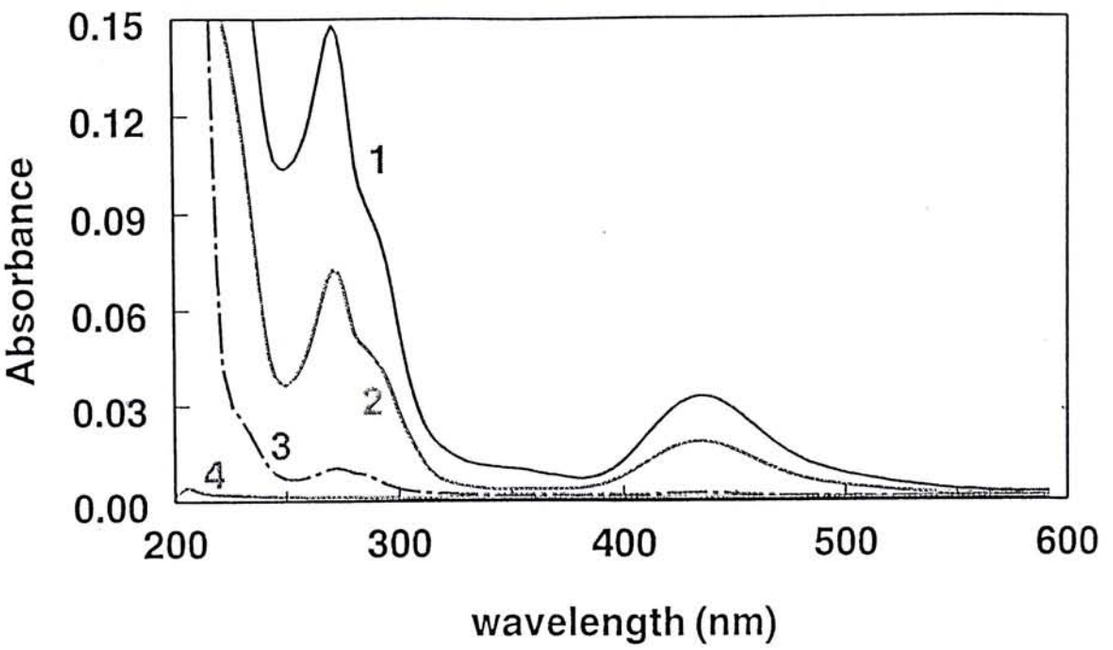

Figure 22c. Absorption spectra of the peaks showed in figure 22b.

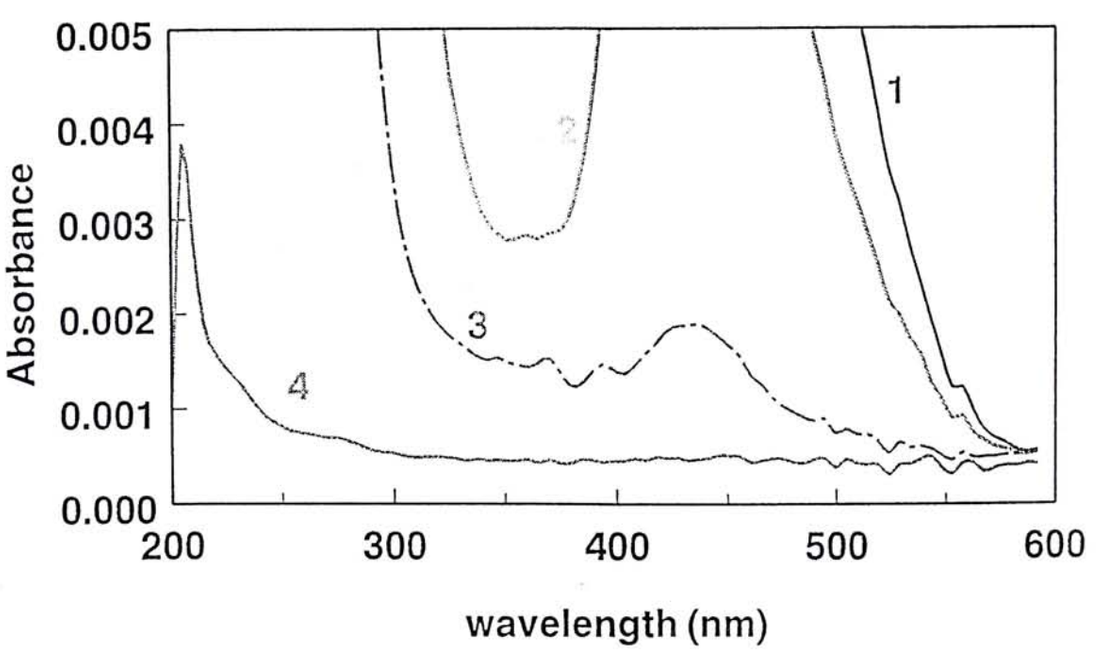

Figure 22d. Highly magnified (x 30) absorption spectra of the peaks showed in figure 22c.

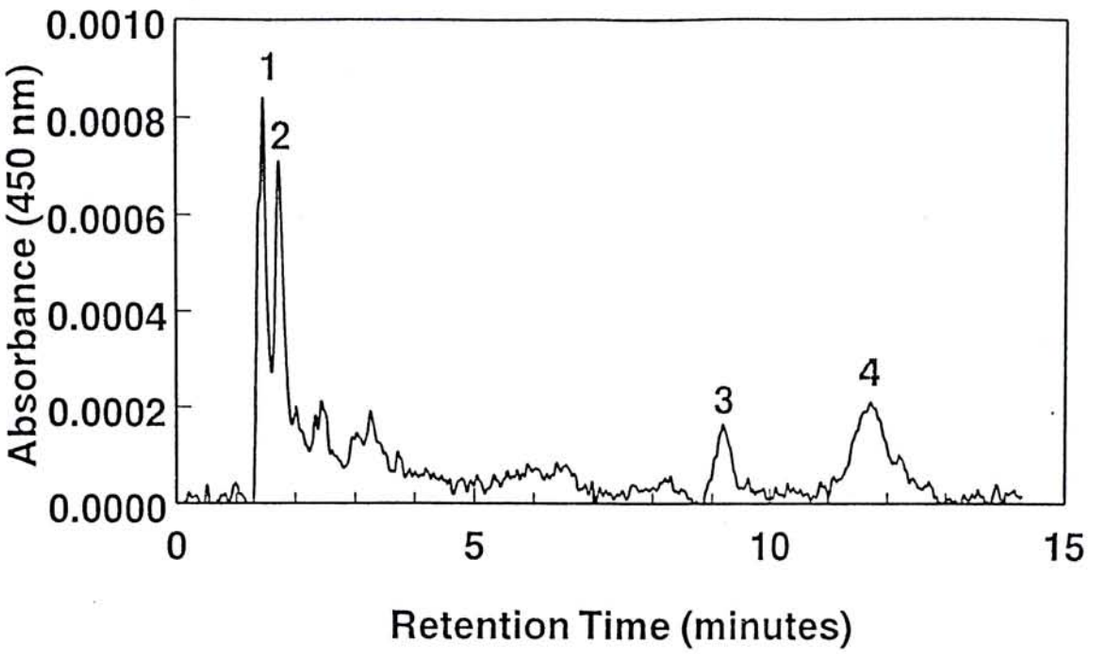

Figure 23a. Chromatogram of serum from rats fed with astaxanthin (1mg/kg) at 450 nm.

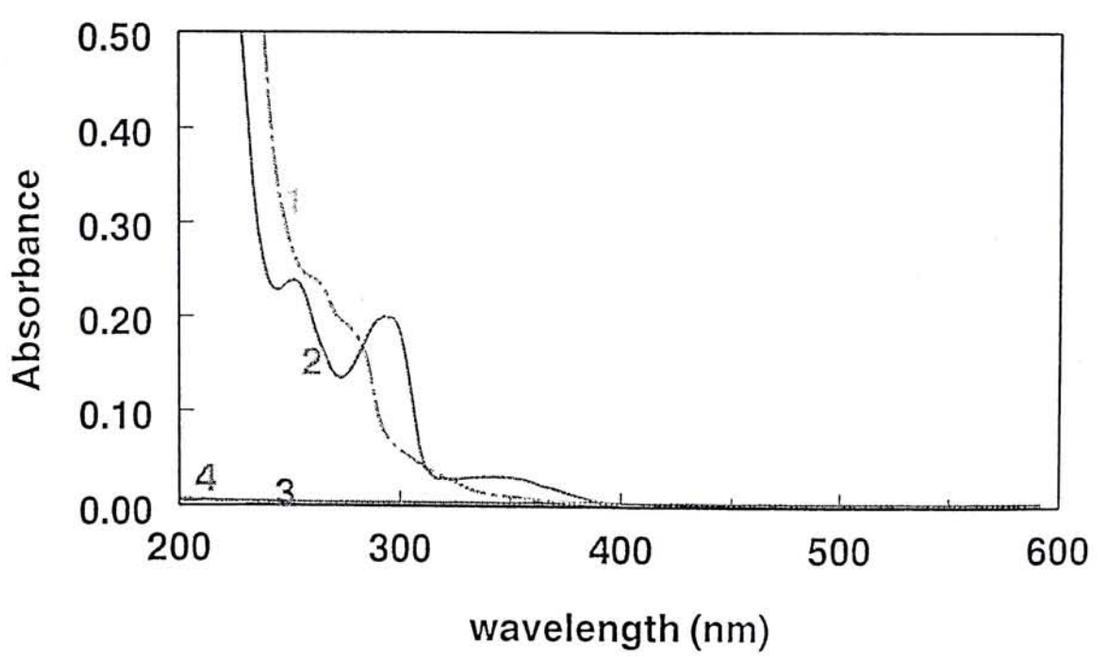

Figure 23b. Absorption spectra of the peaks showed in figure 23b.

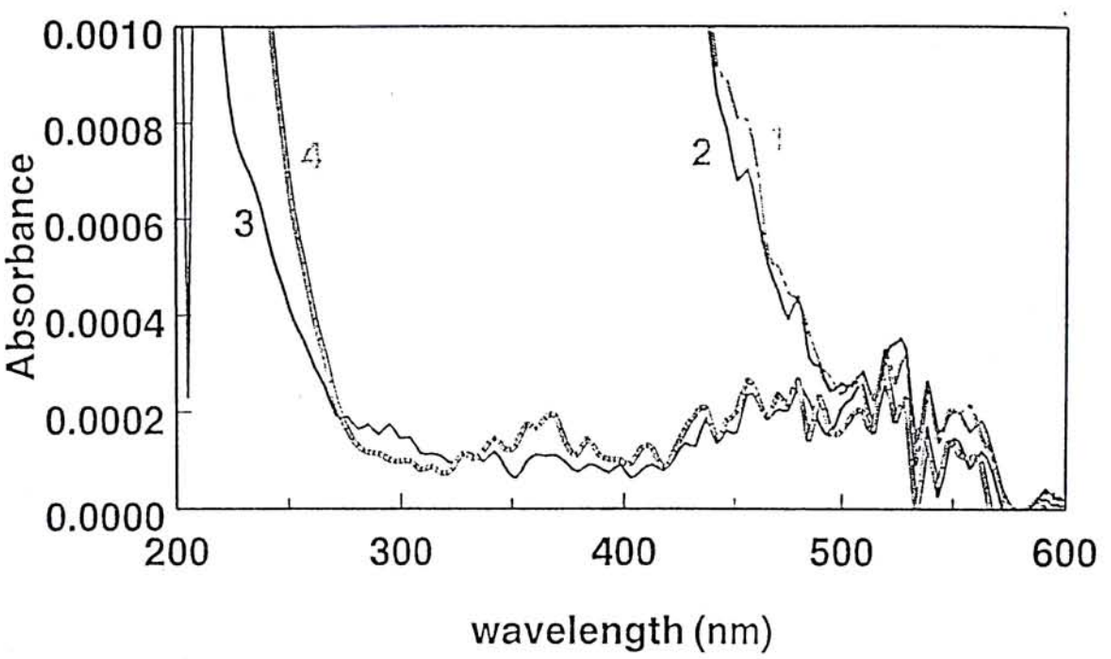

Figure 23c. Highly magnified (x 500) absorption spectra of the peaks showed in figure 23b.

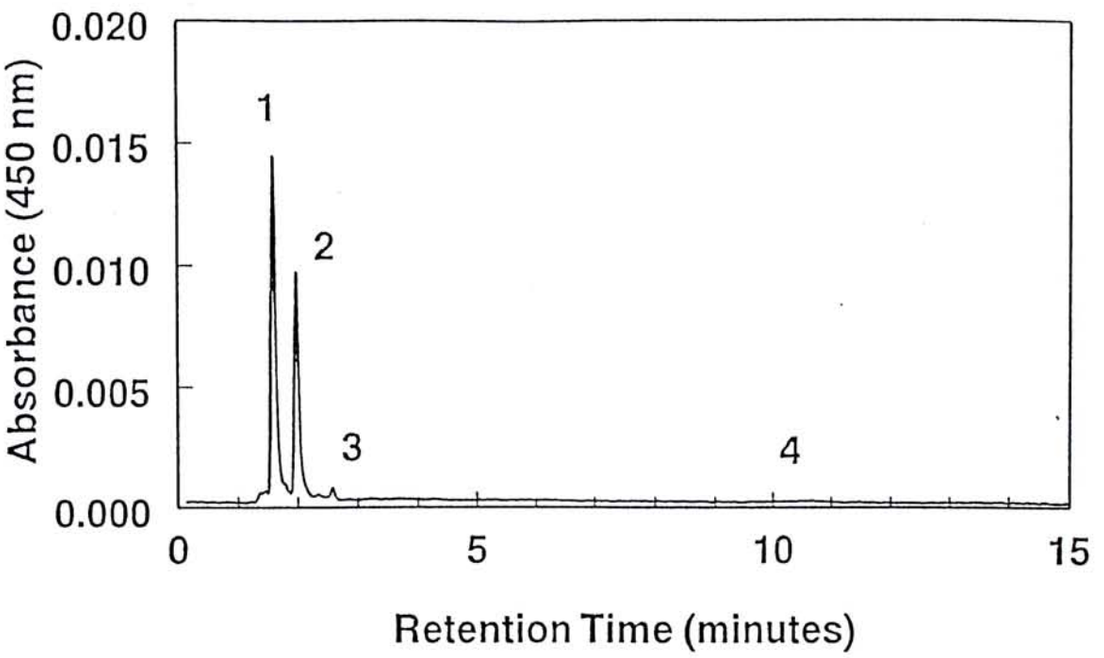

Figure 24a. Chromatogram of serum from rats fed with astaxanthin (10mg/kg) at 450 nm.

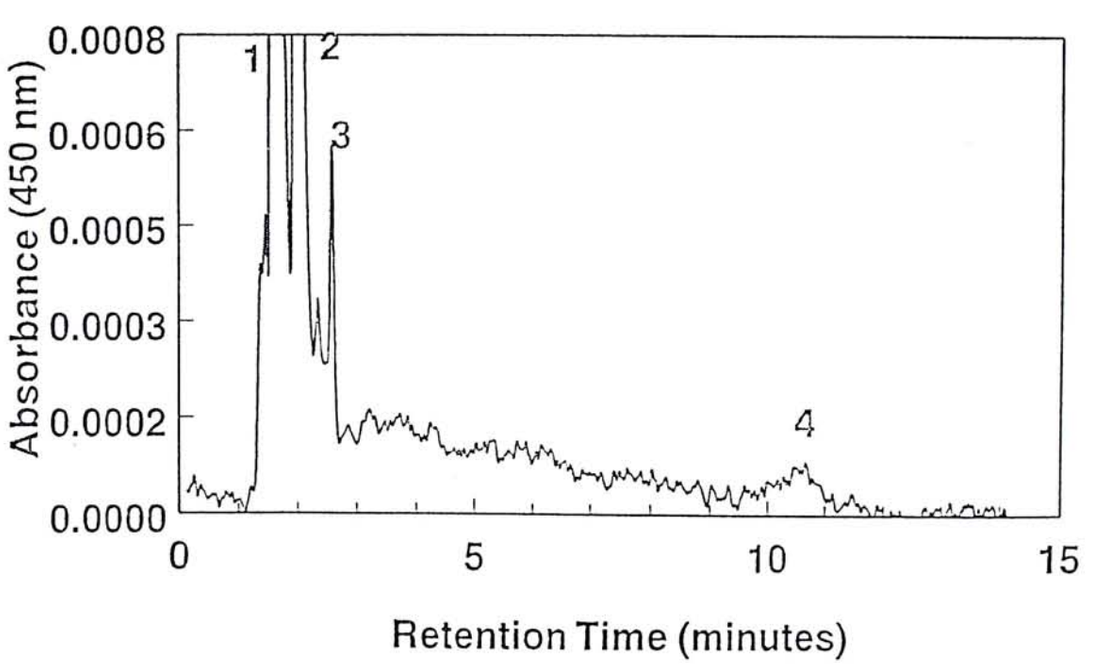

Figure 24b. Highly magnified chromatogram (x 25) of the absorption peaks showed in figure 24a

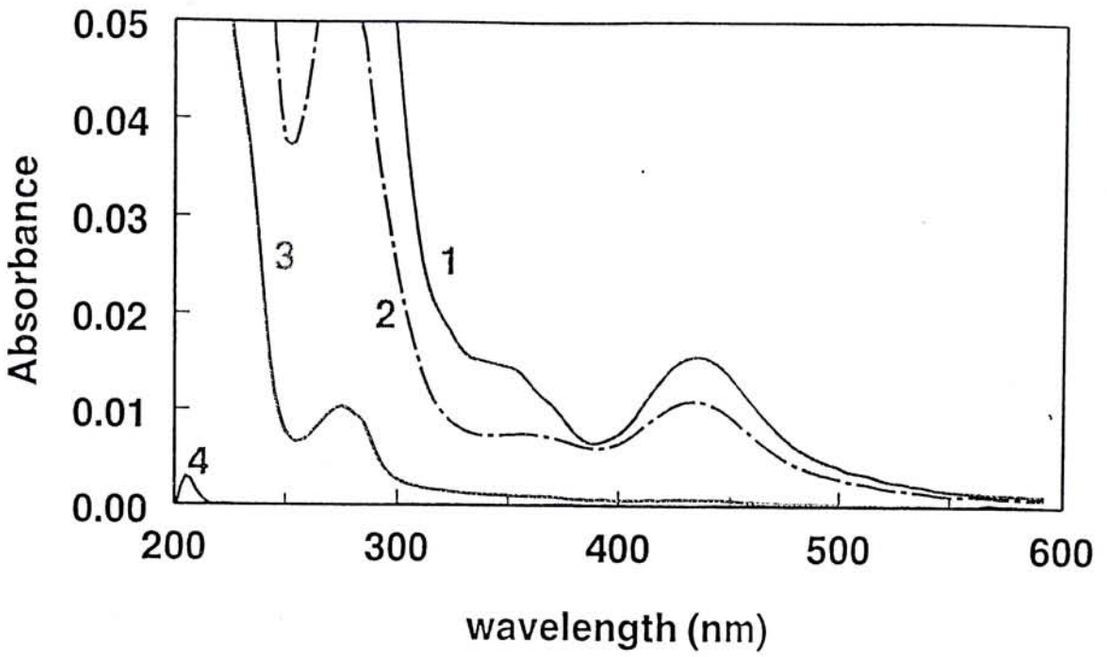

Figure 24c. Absorption spectra of the peaks showed in figure 24b.

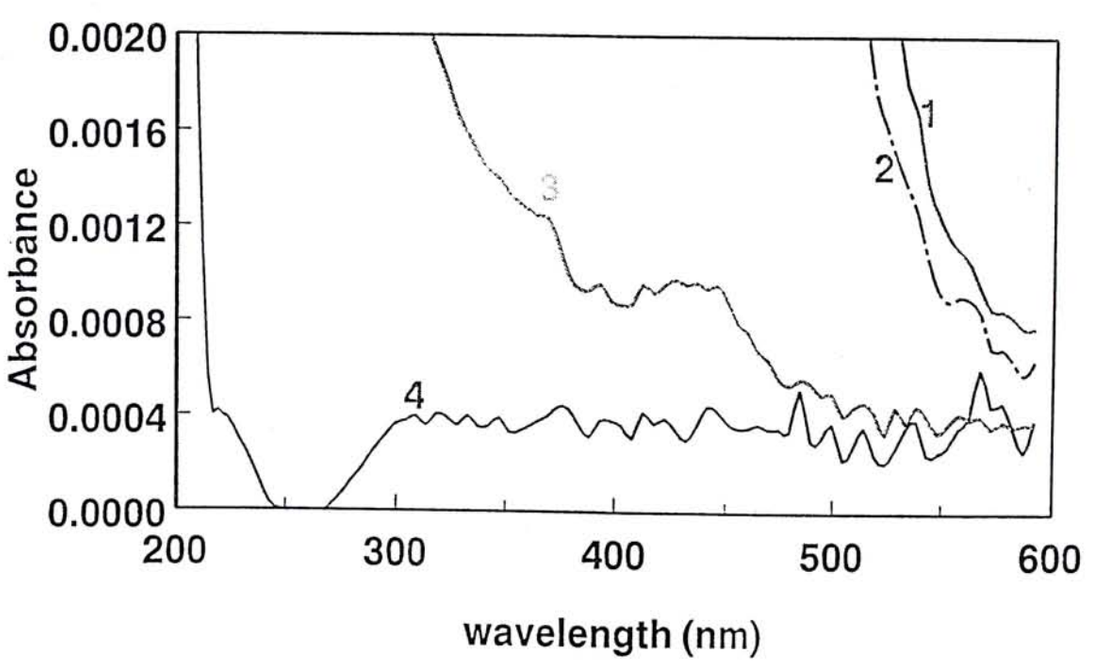

Figure 24d. Highly magnified (x 25) absorption spectra of the peaks showed in figure 24d.

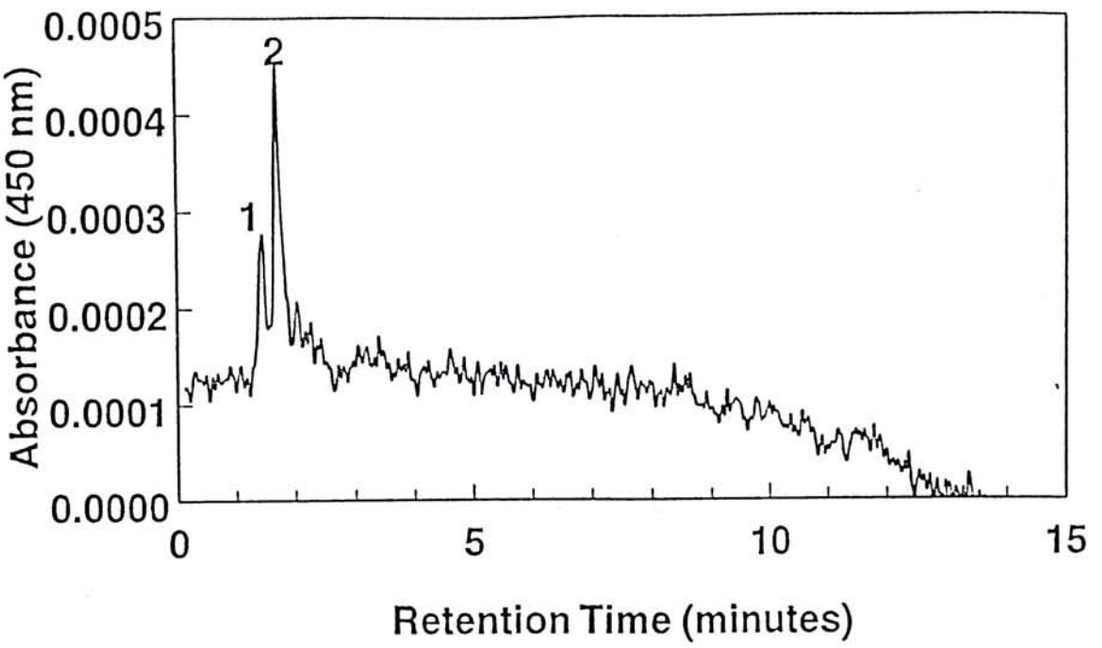

Figure 25a. Chromatogram of retina from rat fed with normal diet at 450 nm.

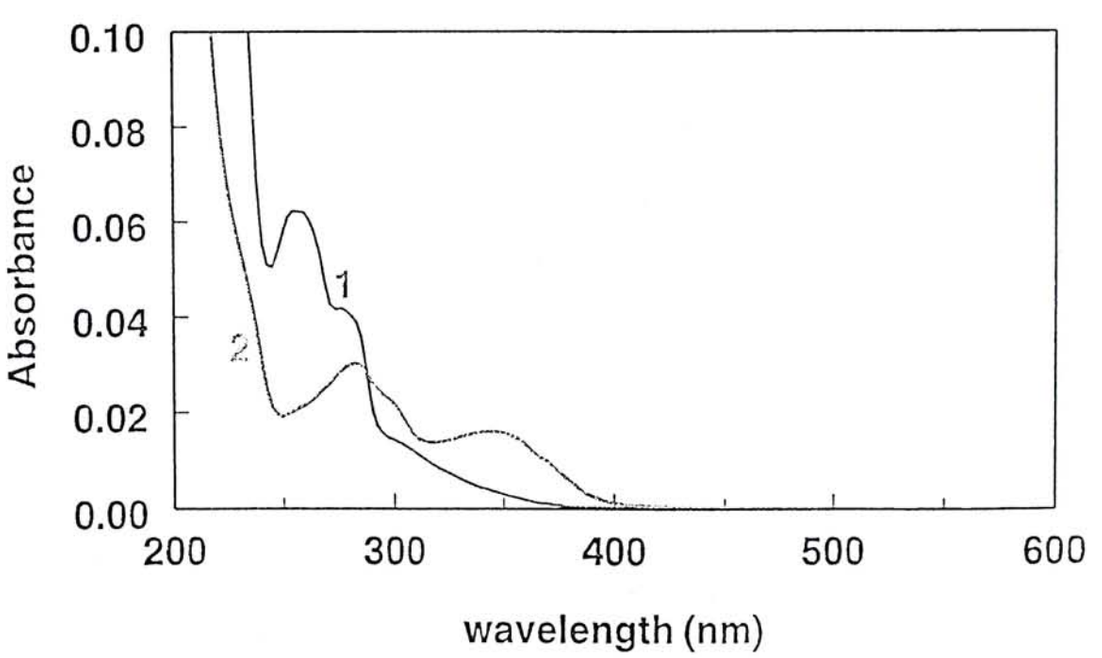

Figure 25b. Absorption spectra of the peaks showed in figure 25a.

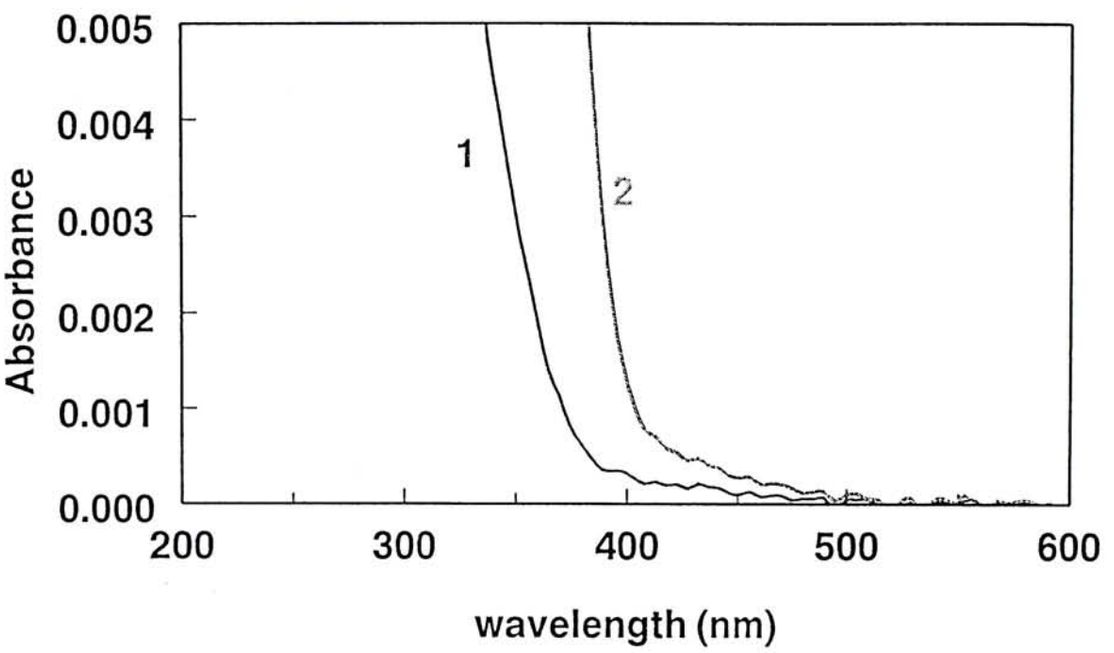

Figure 25c. Highly magnified (x 20) absorption spectra of the peaks showed in figure 25b.

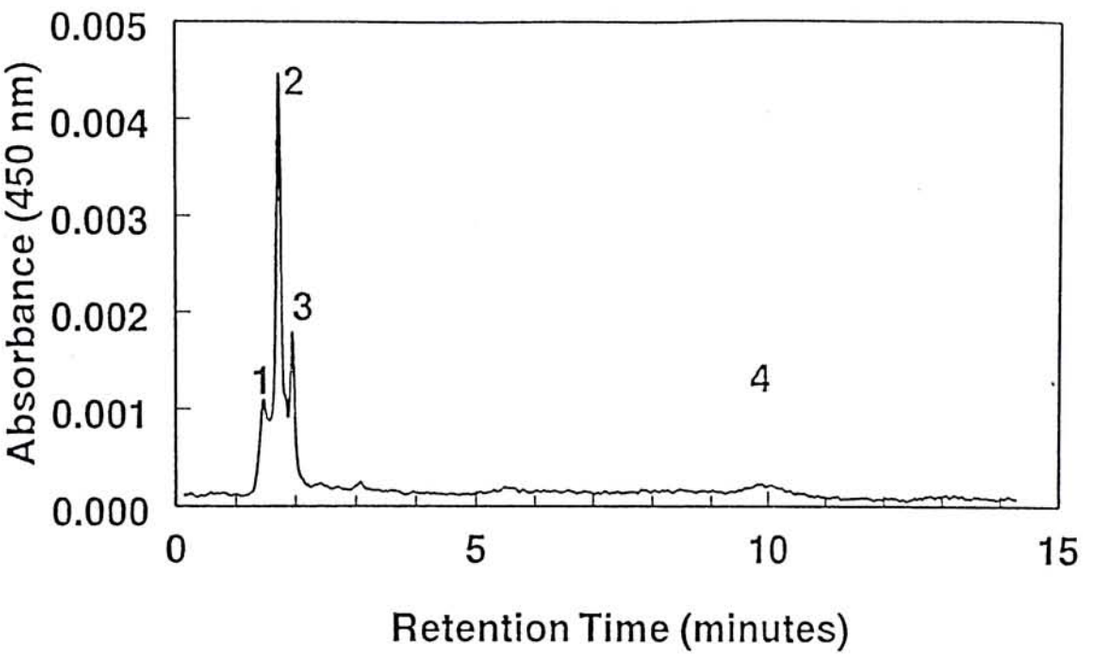

Figure 26a. Chromatogram of retina from rat fed with oil at 450 nm.

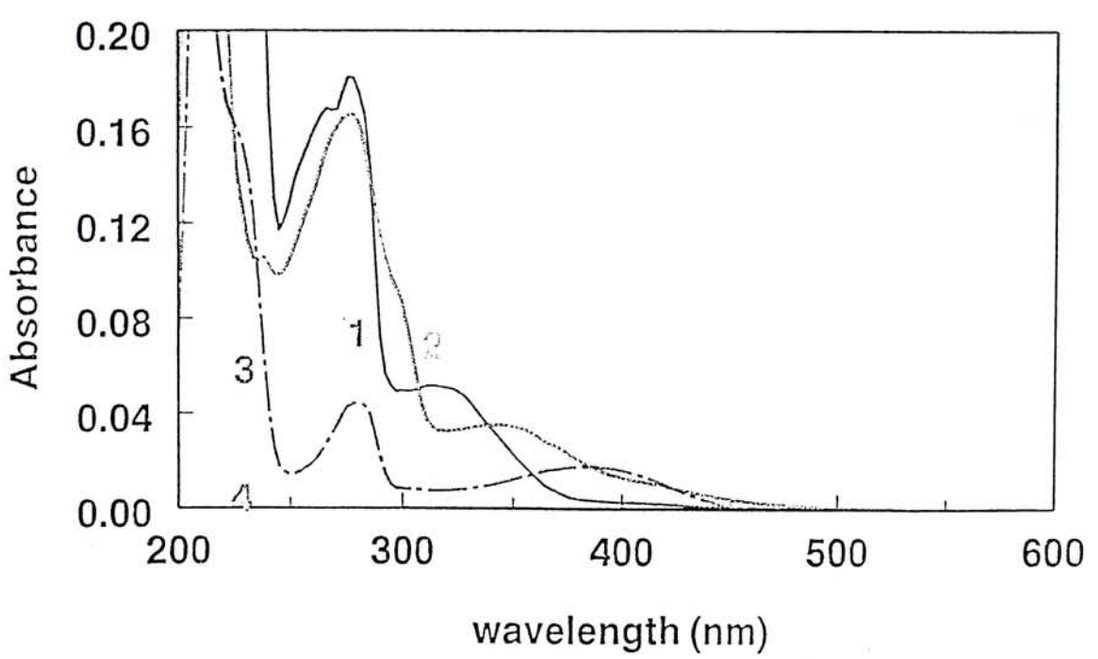

Figure 26b. Absorption spectra of the peaks showed in figure 26a.

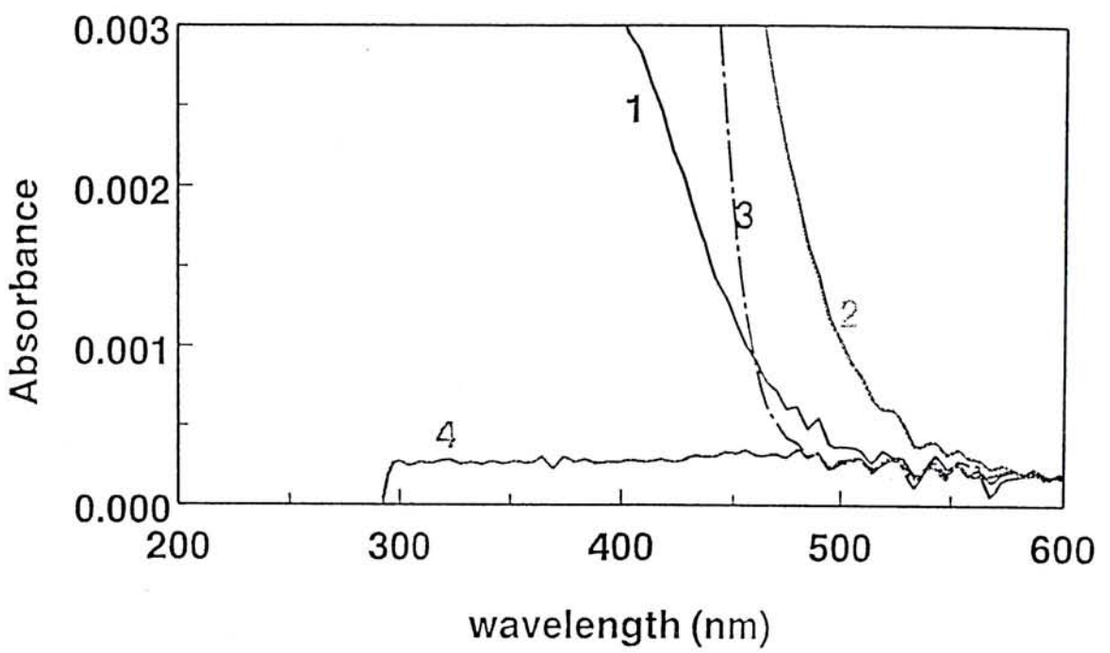

Figure 26c. Highly magnified (x 16) absorption spectra of the peaks showed in figure 26b.

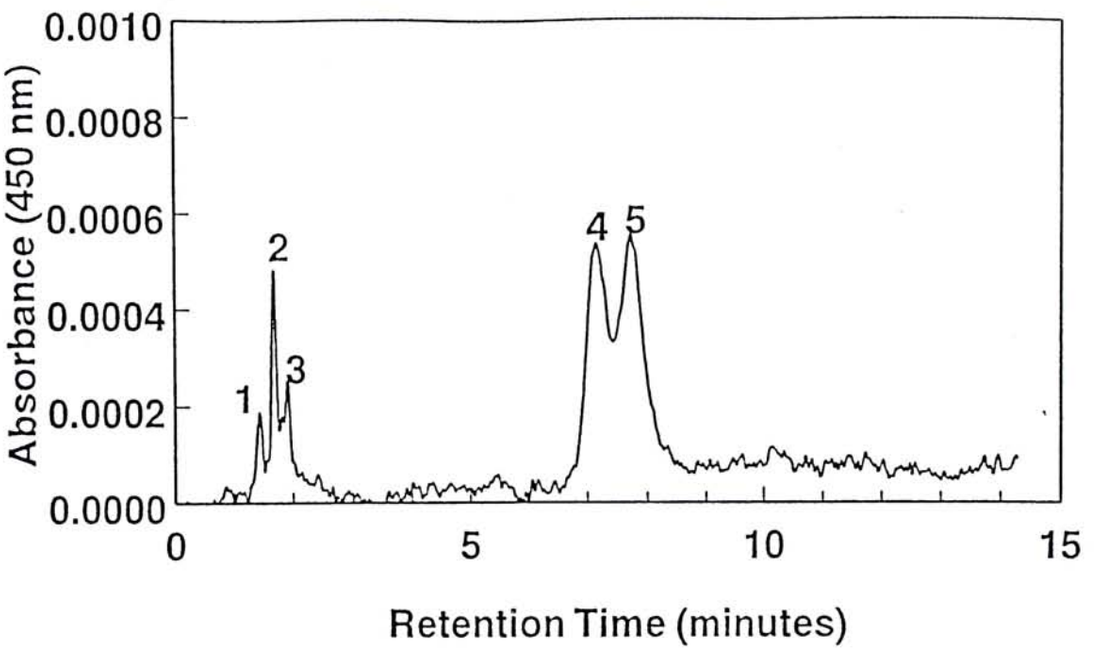

Figure 27a. Chromatogram of retina from rat fed with lutein (1mg/kg) at 450 nm.

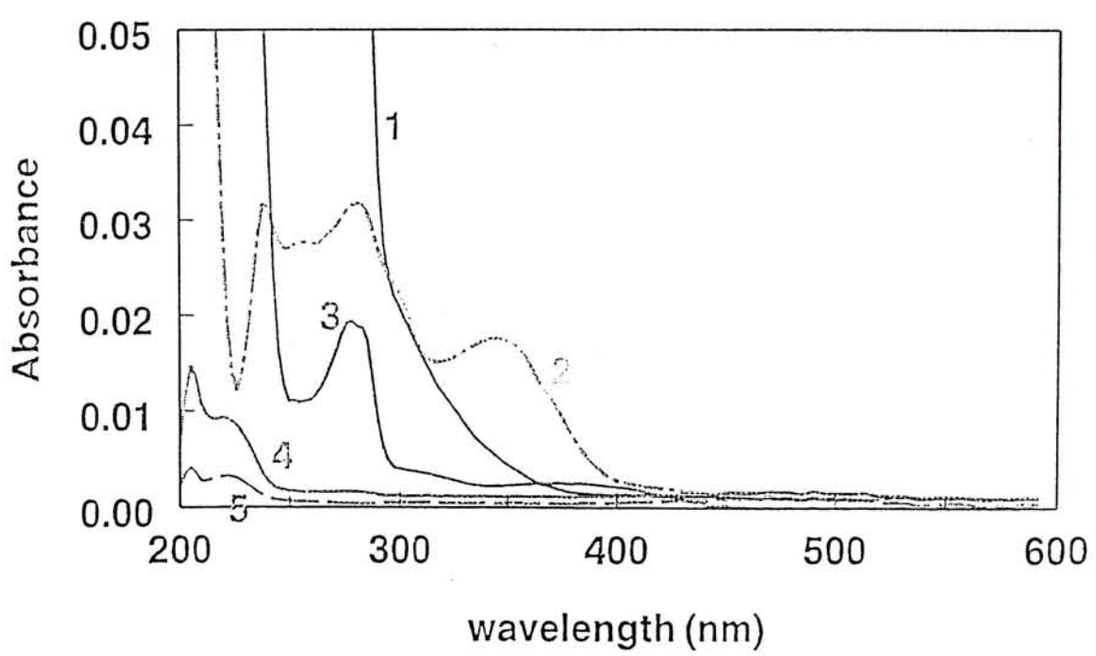

Figure 27b. Absorption spectra of the peaks showed in figure 27a.

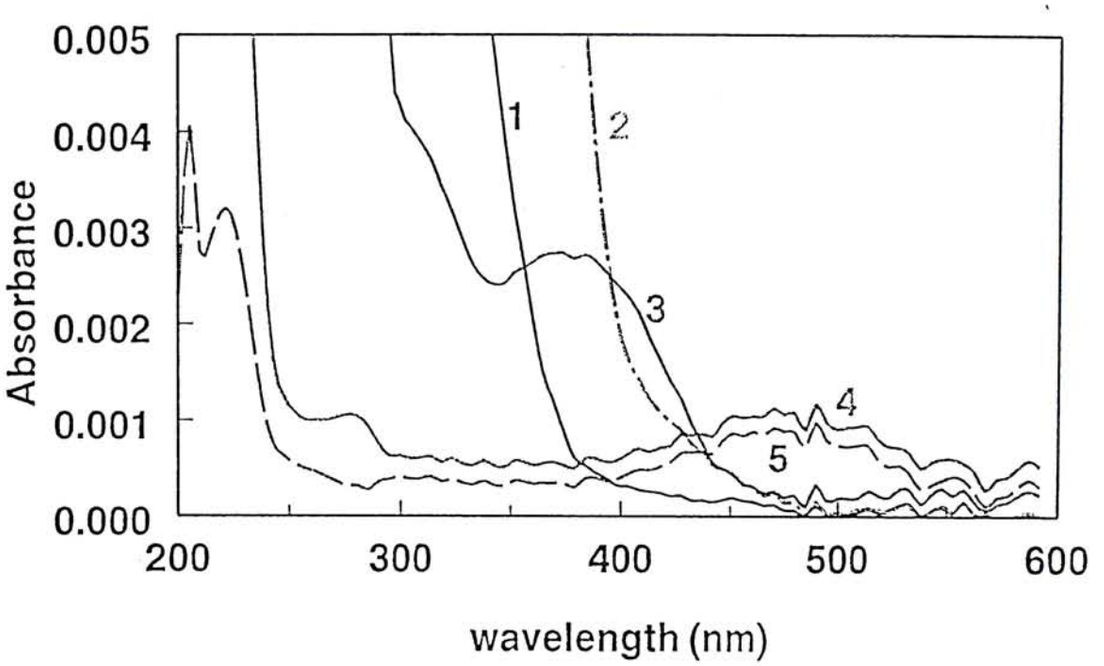

Figure 27c. Highly magnified (x 17) absorption spectra of the peaks showed in figure 27b.

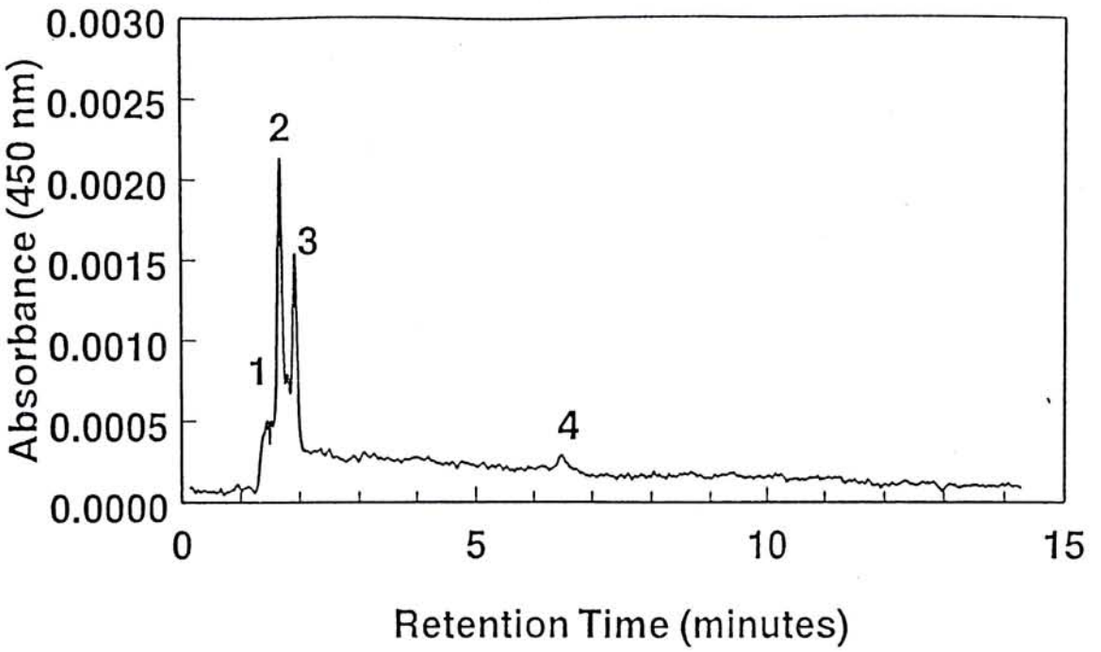

Figure 28a. Chromatogram of retina from rats fed with lutein (10mg/kg) at 450 nm.

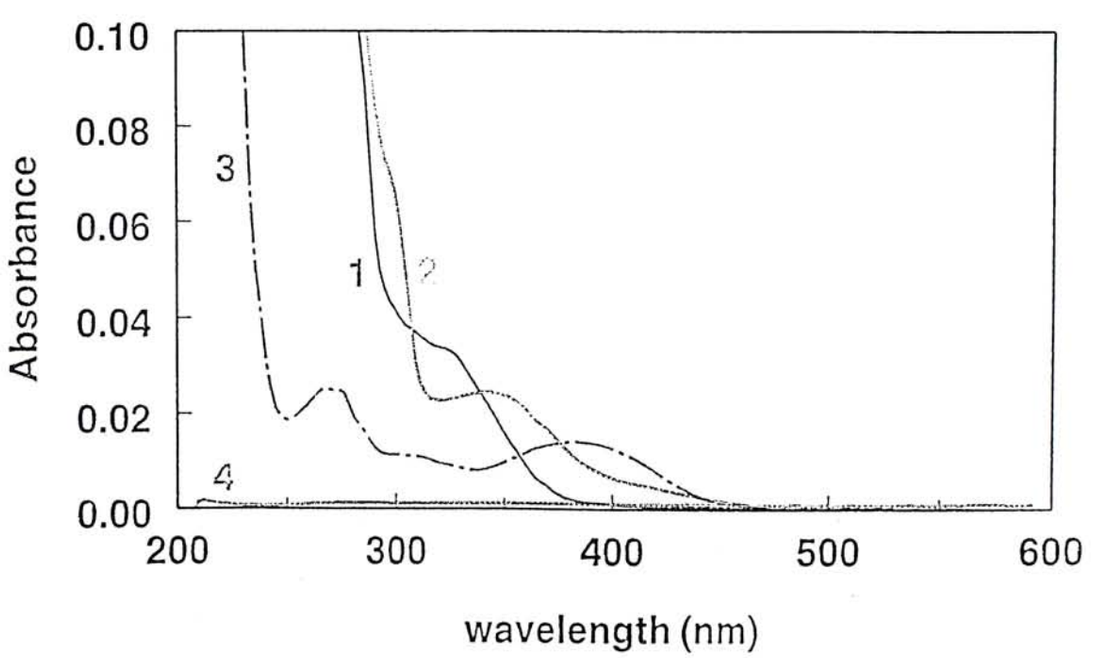

Figure 28b. Absorption spectra of the peaks showed in figure 28a.

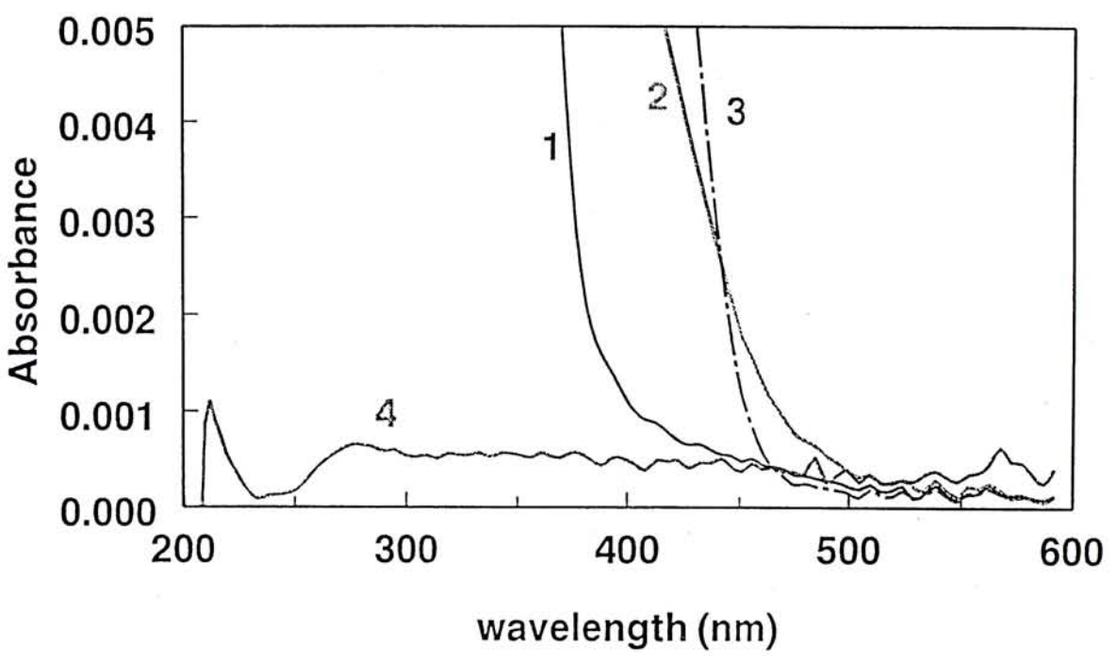

Figure 28c. Highly magnified (x 18) absorption spectra of the peaks showed in figure 28b.

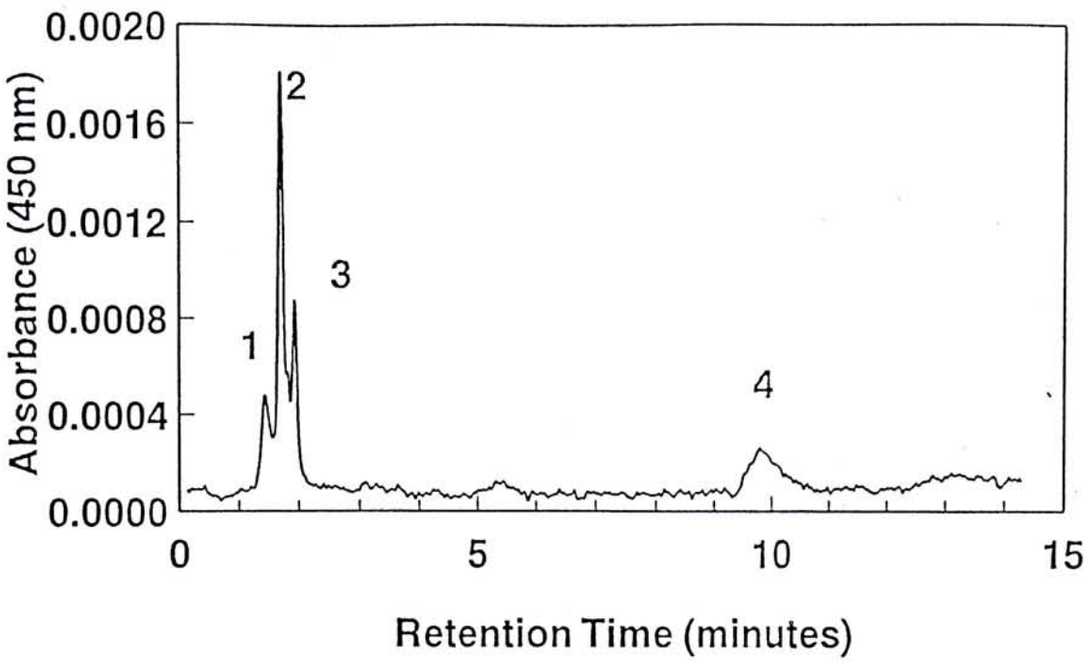

Figure 29a.Chromatogram of retina from rats fed with astaxanthin (1mg/kg) at 450 nm.

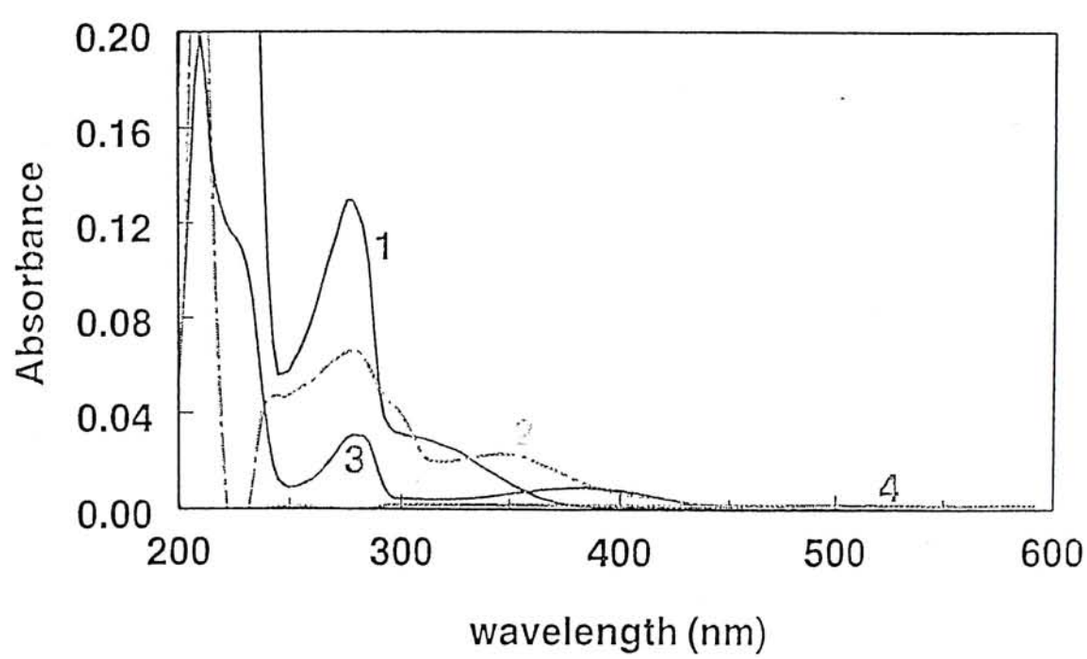

Figure 29b. Absorption spectra of the peaks showed in figure 29a.

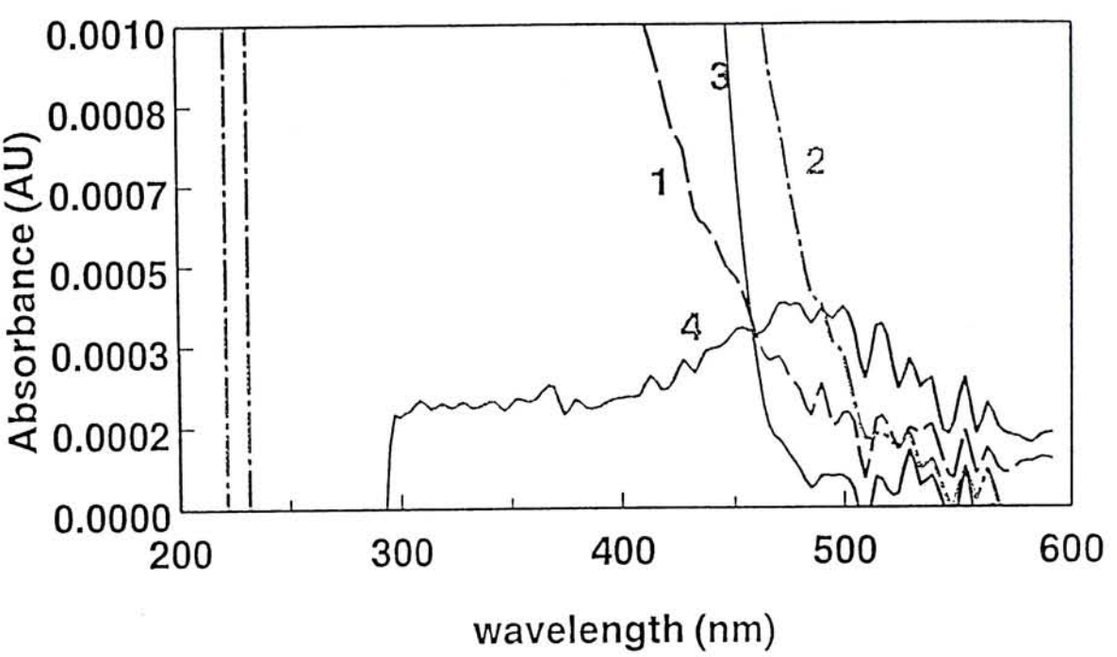

Figure 29c. Highly magnified (x 200) absorption spectra of the peaks showed in figure 29b.

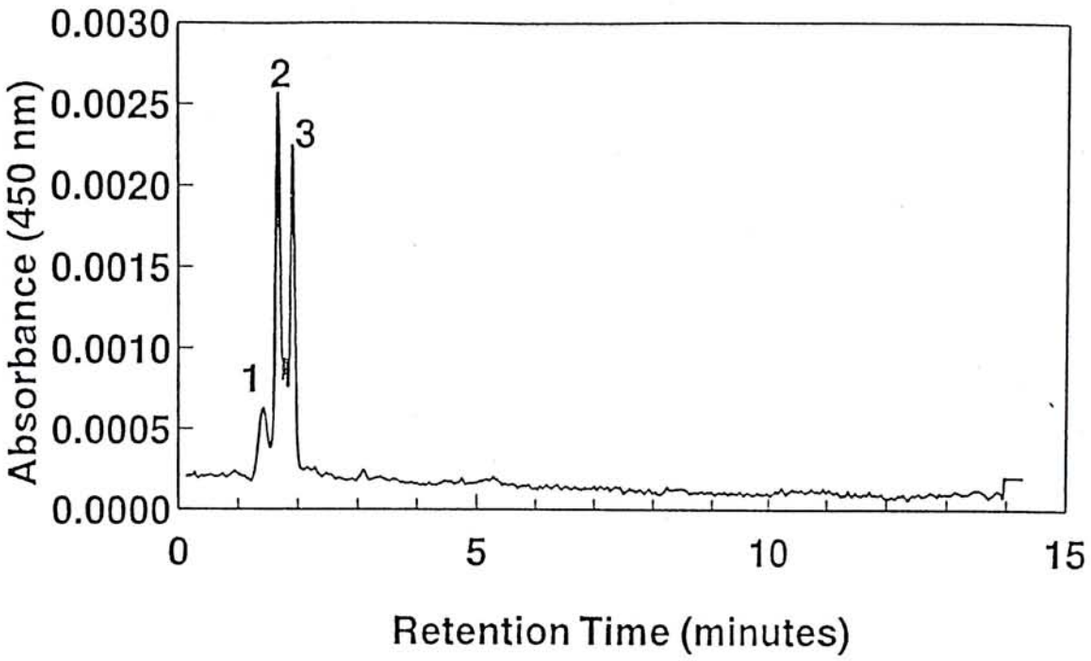

Figure 30a. Chromatogram of retina from rats fed with astaxanthin (10mg/kg) at 450 nm.

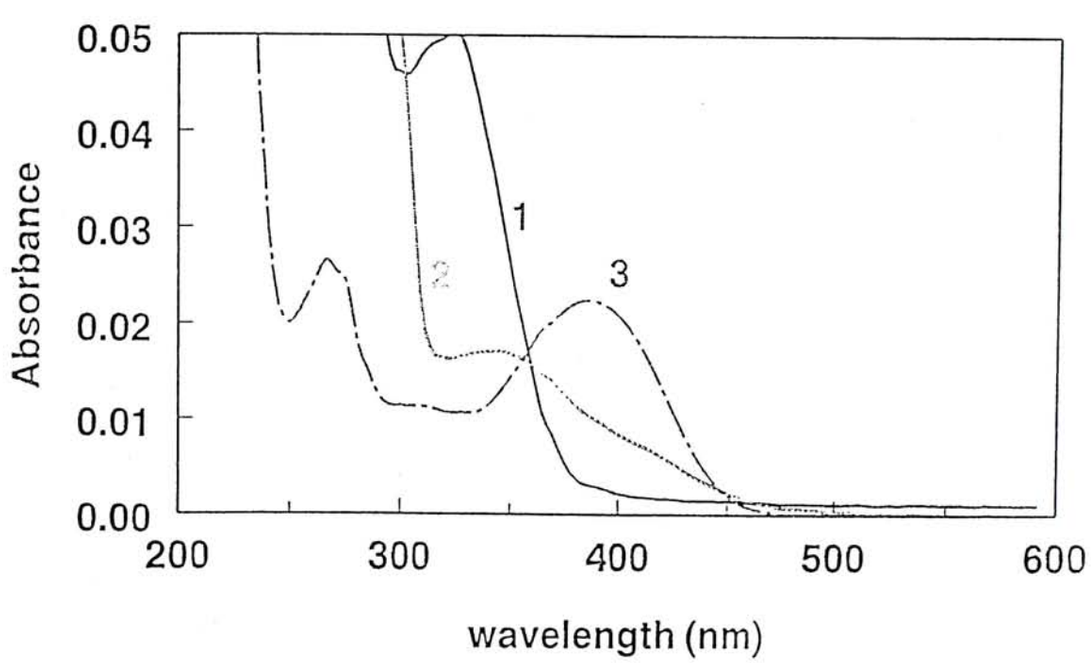

Figure 30b. Absorption spectra of the peaks showed in figure 30a.

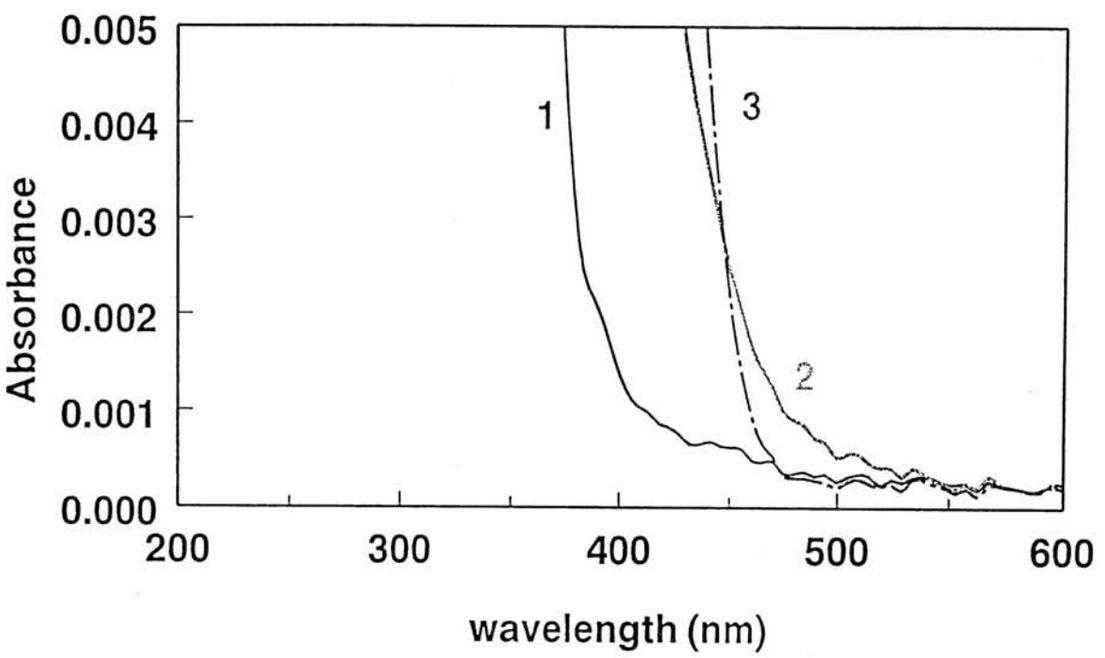

Figure 30c. Highly magnified (x 10) spectra of the peaks showed in figure 30b.

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